

NOCICEPTOR SENSITIVITY IN *DROSOPHILA MELANOGASTER*
LARVAE IS CONTROLLED BY RNA-BINDING PROTEINS

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Abstract
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Currently, there are significant gaps in understanding of the regulatory mechanisms involved in nociceptor sensitivity. Dysregulated nociceptor sensitivity is the likely pathogenesis in many types of chronic pain, a disease that ails over 100 million people in the United States alone. To improve current chronic pain therapies, it is essential to define the regulatory mechanisms responsible for nociception. The goal of this study was to characterize how genes classically involved in RNA processing and translation regulate nociceptor sensitivity. The model organism *Drosophila melanogaster* was used for this study because of their quantifiable response to noxious stimuli and the powerful tools available for genetic manipulations. My results suggest that eukaryotic initiation factors (eIFs) and components of the exon junction complex (EJC) control nociceptor sensitivity by regulating RNA processing and translation, suggesting a major role for RNA metabolism and translation in controlling nociceptor function. Nociceptor-specific knockdown of EJC factors and eIFs resulted in defective thermal and mechanical nociception. No direct link was found between nociceptor dendritic morphology and nociception defects, which indicates that nociceptor morphology does not determine nociceptor sensitivity. Thus, the major findings of

this project revealed that *Drosophila* nociceptor sensitivity is controlled by RNA processing mechanisms from transcription to translation.

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Dedication

This thesis is dedicated to Shem Blackley and Buckley (my furry pal/polar bear/wolf/dog), the lights of my life. Both of your unwavering support, humor, and wills to play outside have brought me so much happiness and inspiration throughout this project. Thank you, Buckley, for sharing my excitement when I talk about science (even if it is just because I am animatedly talking to you). Thank you, Shem for endlessly talking science, theory, and everything in-between with me.

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INTRODUCTION

Currently, there are significant gaps in comprehension of the complex mechanisms responsible for regulation of sensory neuron sensitivity and the pathogenesis of chronic pain. In 2011, the Institute of Medicine (IOM) reported that over 100 million people in the United States suffered from chronic pain and estimated the associated costs to be between \$550-600 billion each year (Institute of Medicine Committee, 2011). The 2011 IOM report designed a plan to improve patient's pain conditions and associated costs by emphasizing preventative care and research into novel pain mechanisms to design effective therapies. Later in 2017, the U.S. Department of Health and Human Services (HHS) declared the widespread misuse of opioid pain relief medication as a public health emergency and prioritized pain research as part of the 5-point strategy to combat the opioid crisis (Azar and Giroir, 2017). Rigorous research of pain neurobiology is of the utmost importance to secure better quality of life and health to those affected by chronic pain and the opioid epidemic.

Throughout evolution, responses to harmful (noxious) stimuli have been modified in response to strong environmental selective pressures to ensure organismal survival. When a noxious stimulus contacts the epidermal layer, polymodal somatosensory neurons known as nociceptors are activated to produce an immediate response in attempt to escape the harmful stimulus (Woolf and Ma, 2007). This process is known as nociception and can be evoked by noxious stimuli such as harsh UV light, erosive chemicals, extreme temperatures, and sharp or heavy objects (Woolf and Ma, 2007). When an environmental insult does cause tissue damage, nociceptors that receive input from the area of damaged tissue are altered to become more responsive to the environment (Bessou and Perl, 1969). Sensitized nociceptors have more intense and exaggerated responses to noxious stimuli (hyperalgesia) and are also

activated by innocuous stimuli during damaged tissue states (allodynia) (Dubin and Patapoutian, 2010). This process is adaptive and allows the area to heal while preventing further damage. However, this process is maladaptive if nociceptor sensitivity does not return to baseline after the injury heals, which results in chronic pain states.

Though the *complete* picture of how nociceptors are sensitized on a cellular and molecular level is not yet clear, regulation of gene expression has been demonstrated to be at the root of the process. There are several points of gene expression regulation. Gene expression is regulated by transcription, inhibition of transcription, and post-transcriptional events. Proteins that regulate ribonucleic acid (RNA) processing, metabolism, subcellular localization, and translation control nociceptor sensitivity at basal and hypersensitive states (Barragan-Iglesias et al., 2018; Dyson and Bellemer, 2017; Jimenez-Diaz et al., 2008; Khoutorsky et al., 2016; Melemedjian et al., 2010; Moy et al., 2017). These observations are consistent with the principle that neural function and plasticity critically relies on RNA-protein interactions for messenger RNA (mRNA) stability, mRNA transcript localization, and temporal control of protein synthesis (Bramham and Wells, 2007; Costa-Mattioli et al., 2009; Jung et al., 2014; Krichevsky and Kosik, 2001; Tsokas et al., 2007). Disruption of any mechanism in RNA regulation may have a negative effect on gene expression, which in turn may have a negative effect on neural function and plasticity (Martin and Ephrussi, 2009). Accordingly, nociceptor sensitivity is controlled by RNA regulation of gene expression; thus, when RNA regulation is disrupted, nociceptor function is defective.

To better understand human pain neurobiology, *Drosophila* is a powerful model organism for investigating the mechanisms responsible for nociceptor function and sensitivity. Not only is the *Drosophila* nervous system well characterized and genetically

conserved with higher order eukaryotes, but the rapid life-span of *Drosophila* and robust genetic tools enable researchers to investigate molecular mechanisms significantly faster than mammalian model organisms (Pandey and Nichols, 2011). When *Drosophila* larvae are contacted by a noxious stimulus, their innate defensive response is to roll along their longitudinal body axis in attempt to escape the threat (Hwang et al., 2007; Tracey et al., 2003). This behavior has been coined the nocifensive escape locomotion (NEL) and is produced normally in nature (Hwang et al., 2007; Zhong et al., 2012). Because this behavior is highly stereotyped and is an innate survival response, it is used to quantitatively determine which genes regulate nociceptor sensitivity and function through genetic techniques.

The sensory circuit for noxious stimuli in humans is analogous to the sensory circuit for noxious stimuli in *Drosophila* larvae. In humans, somatosensory neurons responsible for the detection of tactile signals from internal and external environments originate in the epidermal region of the peripheral nervous system (PNS) and project to the dorsal root of the central nervous system (CNS) in a modality-specific pattern (Todd, 2010). Of the human somatosensory neurons, human nociceptors are anatomically and biochemically distinct from other classes of somatosensory neurons in that nociceptors have the smallest axonal diameters, are either lightly myelinated or unmyelinated, and express receptors and ion channels that are specific for the detection of noxious stimuli (Kumazawa et al., 1996). In *Drosophila* larvae, multidendritic (md) somatosensory neurons responsible for the detection of sensory input originate in the epidermal region and project to the neuropil of the ventral nerve cord of the CNS in a modality-specific pattern (Grueber et al., 2007; Merritt and Whittington, 1995). The multidendritic sensory neurons can be further classified into 4 different classes that are associated with dendritic morphology and the nature of the

activating stimuli (Grueber et al., 2003; Tracey et al., 2003). Of the *Drosophila* larval md neurons, the class IV md (md-IV) neurons function as nociceptors which have the most complex dendritic extensions and express noxious-detecting ion channels (Hwang et al., 2007; Tracey et al., 2003; Xiang et al., 2010). In both human and *Drosophila* sensory systems, nerve impulses generated by activated nociceptors travel to the CNS (ventral nerve cord in flies, spinal cord in humans) (Singhania and Grueber, 2014; Todd, 2010). In the CNS, nociceptors synapse with secondary neurons that transmit the signal to higher-order processing centers in the brain for sensory integration (Singhania and Grueber, 2014; Todd, 2010).

The coding regions of the *Drosophila* genome are 75% homologous with disease-causing genes of the human genome, which makes *Drosophila* a strong tool for advances in clinical research (Ong et al., 2015). Though *Drosophila* and mammals only share approximately >50% nucleotide or codon identity between homologs, 85% of functional domains are conserved between homologs (Pandey and Nichols, 2011). Relevant to this thesis, the functional domains of countless RNA-binding proteins and eukaryotic initiation factors (eIFs), which are the proteins that control protein synthesis, are highly conserved from *Drosophila* to humans (Burd and Dreyfuss, 1994; Marygold et al., 2017).

Mechanisms of Nuclear mRNA Processing

In eukaryotic cells, mature mRNA must be exported into the cytoplasm from the nucleus before translation can occur (Bohnsack et al., 2002; Jung et al., 2014). Before export, mRNA transcripts must undergo 3 co-transcriptional processing steps: 1) addition of the 5' 7-methylguanylate (m⁷G) cap, 2) addition of 100-300 adenylic acid bases by poly(A) polymerase to create the 3' poly(A) tail, and 3) removal of introns by the spliceosome in

splicing and alternative splicing (Sonenberg and Hinnebusch, 2009). The 5' m⁷G cap is required for cap-dependent translation initiated by the 5' cap-binding protein, eIF4E. The 5' m⁷G cap is required for nuclear export and if the 5' m⁷G cap is ever removed, mRNA decay is immediately initiated (Chen and Shyu, 2011).

The poly(A) tail controls mRNA stability, translation efficacy, and mRNA metabolism (de la Pena and Campbell, 2018). The length of poly(A) tails determine the rate of degradation of the mRNA transcript in the cytoplasm; longer poly(A) tails take longer to degrade through deadenylation (Chen and Shyu, 2011; Kornfeld et al., 1989). Longer poly(A) tails are considered to increase transcript stability and translational efficacy through poly(A)-binding protein (PABP) activity (Bi and Goss, 2000; Imataka et al., 1998; Wells et al., 1998). PABP binds to the 3' poly(A) tail and modulates mRNA circularization through protein-protein interaction with the 5' translation initiation complex to decrease the chance of ribosomal disassembly after each round of translation (Imataka et al., 1998; Wells et al., 1998). Therefore, long poly(A) trails increase transcript stability and translation efficiency by forming a closed circular mRNA “track” and by providing more surface available to support multiple protein-protein interactions (Sonenberg and Hinnebusch, 2009).

Alternative splicing of one pre-mRNA has the ability to create multiple (sometimes thousands) of unique mRNA transcripts, which increases protein diversity and enables specialized cell functions (Park and Graveley, 2007). Though the removal of introns is executed by the primary spliceosome complex, human and *Drosophila* SR proteins and exon junction complex (EJC) core proteins define exon boundaries by binding to splice- and branch-site sequences of introns (Barbosa et al., 2012; Black, 2003; Hayashi et al., 2014; Singh et al., 2012). SR- and EJC-modulated exon definition directs the exons that the

spliceosome will incorporate into the final mRNA. *Drosophila* pre-mRNAs that contain long introns, such as *mitogen activated protein kinase (mapk)*, are targeted for EJC-modulated splicing whereas loss of EJC core components results in dramatic exon skipping and reduced transcript levels (Ashton-Beaucage et al., 2010; Roignant and Treisman, 2010).

Tissue-specific RNA-sequencing data demonstrated that the *Drosophila* nervous system has the greatest transcriptome complexity with multiple genes subject to dramatic RNA-processing events that result in over 10,000 different functional proteins (Brown, 2014). A great example of a diverse transcript that is regulated by alternative splicing is *down syndrome cell adhesion molecule (dscam)* in *Drosophila*. Of the 115 exons in *dscam*, 95 exons are alternatively spliced to create 38,016 functional transcripts in a mutually exclusive manner (Graveley, 2005; Schmucker et al., 2000). *Dscam* is necessary proper neural connectivity in development and certain isoforms of *dscam* seem to regulate neural wiring patterns in a cell-specific manner (Chen et al., 2006; Neves et al., 2004; Zipursky et al., 2006). This example supports the notion that alternative splicing is a critical process for specialized cell function and connectivity.

Eukaryotic Translation Initiation

There are two modes by which eIFs can initiate translation: cap-independent or cap-dependent translation. Cap-dependent translation initiation requires the activity of specific eIFs of the eIF4 group: eIF4E, eIF4G, eIF4A, and eIF4B (Gingras et al., 1999). The eIF4E protein has a strong affinity for the 5' m⁷G cap of the mRNA transcript and once bound, eIF4E will recruit eIF4G, eIF4A, and eIF4B to form a complex known as the eIF4F complex (Gingras et al., 1999). In the eIF4F complex, eIF4G serves as a scaffolding protein, eIF4A is an ATP-dependent helicase, and eIF4B increases eIF4A activity (Gingras et al., 1999).

eIF4E-directed eIF4F assembly is the rate-limiting step of cap-dependent translation and is only available in low levels under normal cell conditions (Bitterman and Polunovsky, 2015). Because of this, eIF4E cap-binding activity is highly regulated to ensure that protein synthesis rates adequately meet the cell's specific demands for protein levels and metabolism. This is reinforced by the cessation of cap-dependent translation when eIF4E levels are depleted (Bitterman and Polunovsky, 2015). Under eIF4E-depleted conditions or cellular stress events, cap-independent translation can be initiated if the mRNA contains an internal ribosomal entry sequence (IRES) to orchestrate translation by directing the ternary 43S preinitiation complex (composed of the 40S ribosomal subunit, GTP, methionyl-bound initiator tRNA (Met-tRNA_i), and eIF2) to bind directly to the region containing the start codon (Hunter et al., 1977; Komar and Hatzoglou, 2011; Majumdar et al., 2003).

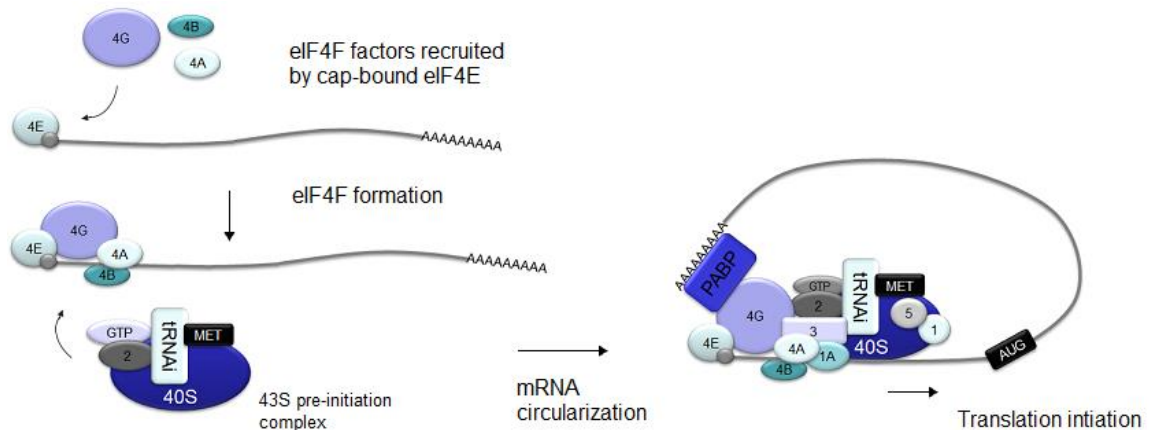


Figure 1. Eukaryotic cap-dependent translation initiation. Formation of the eIF4F complex and recruitment of the 43S preinitiation complex in cap-dependent translation initiation occurs in the cytoplasm.

The formation of the eIF4F complex occurs in the cytoplasm and initiates cap-dependent translation (Figure 1). In the cytoplasm, the eIF4E protein will recognize and bind to the 5' m⁷G cap through the canonical cap-binding motif located in the pocket of eIF4E's concave ventral surface (Gruner et al., 2016). eIF4G contacts the dorsal surface of eIF4E via

the canonical 4E-binding motif (YX₄LΦ; Y= tyr, X= any amino acid, L=leu, Φ= any hydrophobic amino acid) and makes non-canonical contact on the lateral surface of eIF4E (Gruner et al., 2016). eIF4G is a scaffolding protein that coordinates protein-protein interactions and binds to PABP on 3' poly(A) tail to form a circular track, or “closed-loop,” which enables translational machinery to traverse along the mRNA and synthesize protein continuously (Sonenberg and Hinnebusch, 2009). The interaction between eIF4E and eIF4G is thought to be stabilized by the PABP which directly binds to the 3' poly(A) tail (Imataka et al., 1998; Kahvejian et al., 2005). eIF4A is a DEAD box RNA helicase that unwinds the secondary structures of the mRNA strand in an ATP-dependent manner to allow bulky ribosomal complexes to assemble (Li et al., 1999). eIF4B has been shown to simply enhance eIF4A function in RNA helicase activity (Li et al., 1999; Rozen et al., 1990).

The ternary 43S complex is recruited to the 5' untranslated region (5' UTR) of the mRNA strand by the stabilized eIF4F complex and the supporting PABP protein (Sonenberg and Hinnebusch, 2009). When recruited by the eIF4F complex, eIF3 acts as the bridge to connect the 43S complex and eIF4F complex (Lee et al., 2015). Together, the proteins move along the mRNA strand that is opened through eIF4A helicase activity until the start codon is recognized by eIF1 and eIF1A where the anticodon of Met-tRNA_i binds (Gingras et al., 1999; Pestova et al., 1998). Simultaneously upon binding of Met-tRNA_i to the start codon, eIF5 hydrolyzes eIF2-bound GTP which causes a conformational change to accept the 60S ribosomal subunit with eIF5B-GTP once eIF2-GDP, eIF1, eIF3, eIF4F and eIF5 are released (Pestova et al., 2007). The second hydrolysis occurs when the eIF5B-bound GTP, eIF5B-GDP and eIF1A are released, and finally the 80S ribosomal subunit proceeds with elongation (Pestova et al., 2007).

Diversity of eIF4F complex proteins

The mechanism by which cap-dependent translation proceeds has been found to be conserved in eukaryotic organisms; however, there is a high diversity of eIF4F proteins in *Drosophila* (Zuberek et al., 2016). In *Drosophila*, 7 isoforms of *eIF4E* have been identified whereas only 4 isoforms of *eIF4E* have been identified in *H. sapiens* (Marygold et al., 2017). High throughput gene expression profiling determined *eIF4E-1* is ubiquitously expressed throughout larval tissues and is highly expressed in the nervous system, whereas other *eIF4E* isoforms are expressed at residual levels in larval nervous tissue (Gelbart and Emmert, 2013). In *Drosophila*, the 7 *eIF4E* isoforms have various 5' cap binding motifs, eIF4G binding motifs, and phosphorylation sites (Zuberek et al., 2016). Amino acid sequence variation in cap binding motifs of eIF4E isoforms results in various binding affinities and contact sites for the 5' cap (Ghosh and Lasko, 2015; Landon et al., 2014). Of the eIF4Es, eIF4E-3 has the most extensive and stable cap binding pattern whereas eIF4E-4 has the lowest cap binding affinity due to weak specificity for N7 guanosine methylated residues (Osborne et al., 2013; Zuberek et al., 2016).

Though 3 more eIF4E isoforms have been identified in *Drosophila*, human eIF4E isoforms share similar specialized binding and regulatory motifs as *Drosophila* eIF4Es (Zuberek et al., 2016). Here, a comparison of *Drosophila* and human phosphorylation sites is presented. In eIF4E, the serine residue at position 209 (S209) in humans, position 251 (S251) in flies, is a highly regulated phosphorylation site for control of translation rates (Ueda et al., 2004). Protein sequence alignment demonstrated that at least the fly eIF4E-1 and eIF4E-4 isoforms are susceptible to kinase regulation at S251, whereas there is not a serine at position 251 in fly eIF4E-3, rather, there is a rigid proline residue (Figure 2). Notably, human eIF4E-3

does not have phosphorylation site in the C-terminus either (Landon et al., 2014). Only *Drosophila* eIF4E-1 contains all 7 of the eIF4G and eIF4E binding protein (4E-BP) binding motifs (Lasko, 2000). Other *Drosophila* eIF4E isoforms have various combinations of the eIF4G and 4E-BP binding motifs which suggests that each eIF4E isoform makes unique contacts with eIF4G (Lasko, 2000). These variation patterns in *Drosophila* eIF4E biochemical properties mediate the formation of condition- and tissue-specific eIF4F complexes (Lasko, 2000).

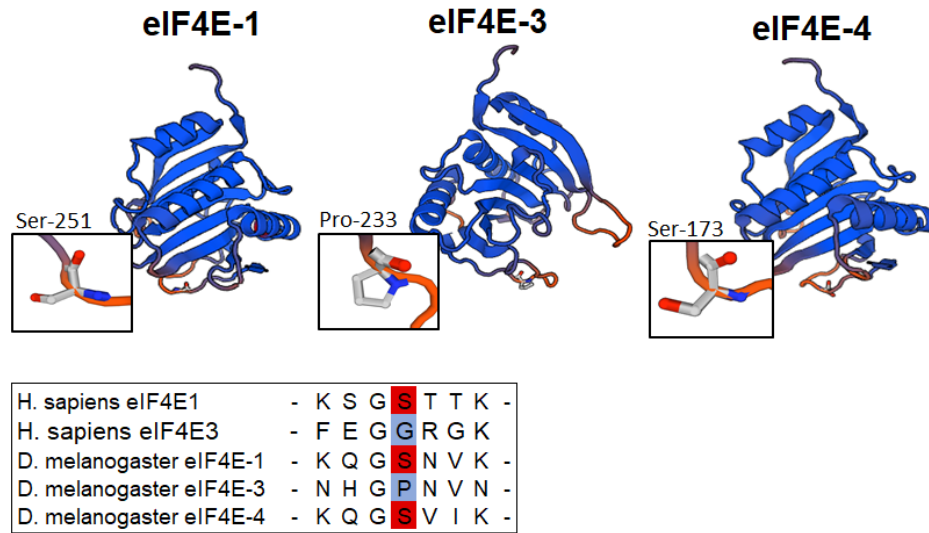


Figure 2. Protein alignment of eIF4E isoforms. Alignment reveals variation in target phosphorylation sites of eIF4E isoforms in humans and flies. Protein alignment constructed in Mega7 using ClustalW alignment. Fly protein models generated using SwissProt.

The eIF4G1 and eIF4G2 isoforms in *Drosophila* only maintain 20-30% identity with human eIF4G orthologs yet both species share the same functional domains that enable eIF4G activity in translation (Bellsollell et al., 2006; Gruner et al., 2016). Human eIF4G2 has a longer N-terminal open reading frame (ORF) and only maintains 70% amino acid identity with the human eIF4G1 isoform which suggests that eIF4G2 has distinct roles apart from eIF4G1 (Imataka et al., 1998). The PABP binding domain and canonical eIF4E binding

domain are located in the N-terminal region and the non-canonical lateral eIF4E binding domain is located in the linker region between the last 2 HEAT domains (Bellssolell et al., 2006; Gruner et al., 2016). Characteristic of most scaffolding proteins, there are multiple HR domains packed within HEAT domains 2-3 at the C-terminus which bind eIF4A and *Drosophila* LK6 (mitogen-activated protein interacting kinase (Mnk) in humans) (Bellssolell et al., 2006). The Mnk-binding domain of human eIF4G 3 aromatic-acidic boxes (AA boxes) which disperse a negative charge across the eIF4G surface to promote Mnk electrostatic interactions (Bellssolell et al., 2006). The MIF4G binding domain located at the central core of eIF4G binds scaffolding protein eIF3 and all eIF4A isoforms and is conserved in all higher eukaryotes (Bellssolell et al., 2006; Li et al., 1999). The central MIF4G domain has also been found in the 4G-like protein Mextli in *Drosophila* and in the non-sense mediated decay (NMD) factor, Upf2 (Hernandez et al., 2013; Ponting, 2000).

There are 2 isoforms of *eIF4A* in *Drosophila* (*eIF4A*, and *eIF4AIII*) and 3 isoforms of *eIF4A* in humans (*eIF4A1*, *eIF4A2*, and *eIF4A3*). All eIF4A isoforms contain RNA helicase characteristic DEAD-BOX and RecA motifs which enable RNA-dependent ATPase and ATP-dependent RNA helicase activities (Hernandez et al., 2004). Of the eIFs, *Drosophila eIF4A* isoforms have the highest conserved nucleotide identity with human orthologs with 74% identity conserved between *eIF4A* orthologs and 88% identity conserved between *eIF4AIII* orthologs (Marygold et al., 2017). Shown in humans, eIF4A is able to bind to the middle and C-terminal motifs on eIF4G, but eIF4AIII is only able to bind to eIF4G at a middle MIF4G domain (Li et al., 1999; Ponting, 2000).

Though eIF4AIII contains similar motifs as eIF4A, eIF4AIII is a core component of the EJC (Li et al., 1999). Later introduced in further detail, the EJC functions in a multitude

of cellular functions like pre-mRNA splicing, NMD, and translation (Boehm and Gehring, 2016). Incorporation of *Drosophila* eIF4AIII into the EJC is directed by the spliceosome factor, Ncampholin (human ortholog is termed CWC22), which interacts with eIF4AIII via a MIF4G binding domain (Barbosa et al., 2012). Spliceosome regulation of EJC formation is evolutionarily conserved throughout eukaryotes (Barbosa et al., 2012).

During the pioneer round of translation in the cytoplasm, eIF4AIII binds directly to Upf3, the NMD factor, at the MIF4G binding domain; this interaction is important for EJC-directed NMD during the pioneer round of translation in mammals, but not flies (Buchwald et al., 2010; Palacios et al., 2004). Functionally distinct from eIF4AIII, eIF4A cannot function in the EJC, it only functions in translation initiation (Andreou and Klostermeier, 2013; Le Hir et al., 2016; Li et al., 1999).

Recent literature has established that isoform-specific eIF4F respond to distinct physiological signals which suggests that the vast array of eIF4F isoforms are not redundant, but instead required for unique physiological responses within the cell (Brown, 2014; Ghosh and Lasko, 2015; Landon et al., 2014; Osborne et al., 2013; Uniacke, 2012). Physiological processes documented to utilize unique eIF4F complexes in *Drosophila* are commonly associated with germ cell differentiation. The eIF4G-like *Drosophila* protein, Mextli, has been characterized to bind to eIF4E-1 and eIF3 in a similar way to eIF4G to promote translation and has been recently found to increase global translational rates in ovarian germ line stem cells (Hernandez et al., 2013). In spermatogenesis, loss-of-function analysis determined that only activity of certain eIF4E and eIF4G isoforms were found to be critical for male fertility: the eIF4E-1 isoform was required in early germ cells for spermatogenesis during which eIF4G and eIF4G2 acted redundantly however eIF4E-1, eIF4E-3, and eIF4G2

were critical later in meiotic divisions of spermatogenesis, but eIF4G was not active (Brown, 2014; Ghosh and Lasko, 2015). In humans, germ cells and cancer cells are associated with translation initiated by isoform-specific eIF4F complexes (Landon et al., 2014; Silvera et al., 2009). Thus, the paradigm of protein synthesis dependence on isoform-specific eIF4F complexes to achieve differential protein expression during changing cell states is applied in multiple different cell types. To determine if this paradigm applies to the changing state of nociceptors during the sensitization process, research focused on the role of eIF4F isoforms in nociception must be conducted.

Mechanisms of mRNA Regulation by the EJC

The exon junction complex (EJC) is a dynamic protein complex that coordinates splicing events of pre-mRNAs, nuclear export, mRNA quality surveillance, mRNA localization, and translation (Ashton-Beaucage et al., 2010; Boehm and Gehring, 2016; Chazal et al., 2013; Ghosh et al., 2012; Giorgi et al., 2007; Le Hir et al., 2016; Le Hir and Seraphin, 2008; Nott et al., 2004; Palacios et al., 2004). The EJC has four core components: eIF4AIII, Tsunagi (Tsu), Mago Nashi (Mago), and Barentsz (Btz), the *Drosophila* orthologs of human eIF4A3, Y14, Magoh, and MLN51, respectively. The EJC is a trimeric core (eIF4AIII, Tsu, and Mago) in the nucleus and a tetrameric core (eIF4AIII, Tsu, Mago, and Btz) before nuclear export and in the cytoplasm (Le Hir et al., 2016). Genome-wide microarray experiments have uncovered that the EJC is active in nearly all cell types and is responsible for the regulation of a wide range of mRNAs (Johnson et al., 2003).

To form the trimeric EJC, eIF4AIII is deposited by the spliceosome factor Nucampholin 20-24 nucleotides upstream of exon junctions after the first step of the splicing reaction (Barbosa et al., 2012; Gehring et al., 2009; Le Hir et al., 2000). The interaction

between Nup60 and eIF4AIII prevents nuclear eIF4AIII ATPase activity before the EJC is formed by keeping eIF4AIII in an open conformation (Barbosa et al., 2012). On the RNA strand, eIF4AIII serves as a platform for EJC core components and peripheral factors by first binding to the ribose-phosphate backbone of pre-mRNAs in a sequence-independent manner (Bono et al., 2006). Tsu and Mago form a heterodimer and bind to eIF4AIII to promote a closed, or “clamped,” confirmation of eIF4AIII on the RNA strand (Bono et al., 2006). Though Tsu has a RNA recognition motif (RRM), crystal structures of human and fly Tsu-Mago heterodimers revealed that Mago completely blocks RRM accessibility of Tsu; therefore, the Tsu-Mago heterodimer relies on the RNA binding property of eIF4AIII for stability (Fribourg et al., 2003; Shibuya et al., 2004).

Once formed on pre-mRNAs in the nucleus, the EJC is a trimeric protein core that tags exon junctions to mediate splicing. EJC-mediated splicing events are critical for transcripts that contain long introns, like *mapk*. Loss-of-function analyses in *Drosophila* have revealed functional transcripts are not generated if EJC core components are not functional (Ashton-Beaucage et al., 2010; Roignant and Treisman, 2010). Before the mRNA is exported into the cytoplasm through the nuclear pore, the EJC forms a tetrameric core (eIF4AIII, Tsu, Mago, and Btz) and forms a compacted messenger ribonucleoprotein particle (mRNP) with multiple peripheral factors that modulate nuclear export, such as the transcription-export complex (TREX) and nuclear RNA export factor 1 (NXF1). Once in the cytoplasm, the tetrameric EJC initiates the pioneer round of translation where it functions as a mRNA quality surveillance complex by recruiting NMD factors, up-frameshift protein factors (Upf1, Upf2, Upf3), in the presence of a preterminal stop codon (Palacios et al., 2004). Though the

function of the EJC was considered to cease after NMD, recent studies suggest that the EJC role in post-transcriptional regulation is far more complex.

The cytoplasmic core EJC factor, Btz, is responsible for multiple post-transcriptional regulatory processes. Btz activity is limited to the cytoplasm where it is involved in EJC stability during the pioneer round of translation, NMD in humans, mRNA localization, and modulation of translation efficacy via interaction with eIF3 (Chazal et al., 2013; Hauer et al., 2016; Palacios et al., 2004; van Eeden et al., 2001). Btz was classically characterized as a critical component of a Staufén-containing mRNP responsible for cytoplasmic mRNA transport and localization of *oskar* in *Drosophila* oocytes (van Eeden et al., 2001). Later in hippocampal neurons, Btz and Staufén were characterized to colocalize in mRNPs at distal dendritic sites—this interaction was RNA-dependent (Macchi et al., 2003). Together, these results suggest that Btz and Staufén have an evolutionarily conserved role in mRNA localization and post-transcriptional regulation in polarized cells, including neurons.

The core EJC components, eIF4AIII and Btz, have been demonstrated to regulate post-transcriptional processes in neurons and influence synaptic plasticity (Fritzsche et al., 2013; Giorgi et al., 2007). There is a MAPK docking site on *stau2* required for dendritic transport of mRNAs in hippocampal neurons (Nam et al., 2008). Btz binds to *stau2* to create a transport mRNP in polarized cells which controls translation in a spatial and temporal manner by holding the mRNA in a translational-inhibitory complex (Macchi et al., 2003; van Eeden et al., 2001). Furthermore, eIF4AIII regulates splicing and NMD of the Activity-regulated cytoskeletal associated protein (Arc/Arg3.1) at distal dendritic sites of hippocampal neurons (Giorgi et al., 2007). EJC-mediated splicing of Arc has recently been

found to enhance translation in an activity-dependent manner in cultured neurons (Paolantoni et al., 2018).

For over a decade it has been observed that translation of spliced transcripts produces higher yields of protein, which may be due to Btz activity (Ma et al., 2008; Nott et al., 2004). Btz biasedly enhances translation of EJC-associated transcripts but does not regulate transcripts that encode ribosomal subunits or transcripts with 5' oligopyrimidine tracts (TOP mRNAs) (Hauer et al., 2016). Unlike the Tsu/Mago heterodimer proteins, Btz and eIF4AIII were found to be associated with monosome fractions and polysome fractions in polysome profile analyses of human immortal cell lines, which indicated that Btz and eIF4AIII may be associated with active translation (Chazal et al., 2013). Furthermore, cytoplasmic levels of Btz and eIF4AIII were found to be stoichiometrically related in immunoprecipitation analyses of human embryonic kidney-derived 293 (HEK293) cells (Chazal et al., 2013). Though exact mechanism of Btz-enhanced translation, nor the role of eIF4AIII in active translation have not yet been clarified, Btz has been shown to interact with eIF3 during translation on spliced mRNAs (Chazal et al., 2013).

Post-Transcriptional Regulation and Metabolism of mRNA

Neurons are polarized cells that contain 3 subcellular compartments: the dendrites, the soma (cell body), and the axon. These 3 subcellular compartments are biochemically distinct and critically dependent on post-transcriptional regulation of mRNA for specialized protein expression and proper function (Kang and Schuman, 1996). To achieve biochemically distinct subcellular regions, RNA-granules and/or mRNPs bind to mRNA to repress translation while they transport mRNA to the correct location in the cell. Repression of translation during mRNA localization enables spatial and temporal control of protein

expression levels in response to extracellular and intracellular signaling (Krichevsky and Kosik, 2001; Martin and Ephrussi, 2009).

Regulatory elements within the 5' UTR and 3' UTR of mRNA transcripts, known as *cis*-regulatory elements, are targeted by *trans*-acting mRNPs for nuclear export, subcellular localization, and local translation (Martin and Ephrussi, 2009). This neuronal phenomenon was first addressed in a study that investigated the *cis*-sequences regulatory elements in the 3' UTR of α -*CaMKII* (Mayford et al., 1996). In hippocampal neurons, transcripts with 3' *cis*-acting cytoplasmic polyadenylation element (CPEs), like α -*CaMKII*, are localized to active dendritic synapses by *trans*-acting CPE-binding proteins (CPEBs) (Huang et al., 2003; Kim and Martin, 2015). To temporally control localized protein expression, 4E-BPs like Neuroguidin or Maskin bind to CPEB and eIF4E to inhibit eIF4F complex formation (Jung et al., 2006).

Because formation of the eIF4F complex drives cap-dependent translation, eIF4F activity is highly regulated by signaling pathways that control gene expression. Intracellular signaling pathways such as mitogen-activated protein kinase (MAPK), or the mammalian target of rapamycin (mTOR) modulate eIF activity through phosphorylation. Neuron homeostasis relies on critical molecular sensors like mTOR to target protein synthetic machinery (eIF4F complex, 43S pre-initiation complex, etc.) to regulate translation in response to homeostatic disruption (Yanagiya et al., 2012).

The mTOR signaling pathway influences protein synthesis through modulation of 4E-BPs which inhibit translation when bound to eIF4E (Hinnebusch, 2012). As previously stated, eIF4G binds to eIF4E at its dorsal and lateral surfaces to promote translation; however, eIF4G competes for eIF4E-binding at these surfaces with 4E-4E-BPs (Hinnebusch,

2012). The rigid proline residues in the canonical binding motif of 4E-BPs give the inhibitory proteins a competitive edge over eIF4G and allow for the structure to bind at the dorsal and lateral surface of eIF4E residues with a stronger affinity (Gruner et al., 2016). The rate at which 4E-BPs compete with eIF4G for eIF4E-binding is dependent on the 4E-BP phosphorylation state: only hypophosphorylated 4E-BPs are able to bind to eIF4E. Additionally, when hypophosphorylated 4E-BPs are not bound to eIF4E, they are targeted for degradation via the ubiquitination and protease pathway (Yanagiya et al., 2012). In response to cytokines and growth factor signaling, mTOR phosphorylates 4E-BPs to activate translation and increase protein levels (Gingras et al., 1999; Nandagopal and Roux, 2015).

This described mode of translation inhibition is not universal for all 4E-BPs. Diverse 4E-BPs can actively repress translation in response to unique cell signals or protein interactions. CYFIP1, a neuron-specific 4E-BP, has been found to associate with FMRP (Fragile X Mental Retardation Protein) in dendrites to specifically repress target mRNAs and regulate synaptic plasticity in a temporal manner (Napoli et al., 2008). Alternatively, the *Drosophila* eIF4G homolog, Mextli, has been suggested to substitute in translation under conditions that render eIF4G inactive (Peter et al., 2015). This is supported by the finding that Mextli bound to eIF4E is resistant to displacement by competitive 4E-BPs, whereas eIF4G can be displaced (Peter et al., 2015). These examples exemplify the complex and diverse regulatory mechanisms that cells utilize to control gene expression for specific functions.

MAPK signaling has a pivotal role in gene expression regulation by controlling RNA processing from transcription to translation, thus MAPK signaling has an essential role in synaptic plasticity regulation (Bramham et al., 2016). At synaptic sites, MAPK signaling

modulates translation of localized mRNA transcripts in an activity-dependent manner (Kelleher et al., 2004). The MAPK phosphorylation cascade culminates on Mnk (fly ortholog is LK6) which phosphorylates eIF4E at S209 (S251 in flies) to drive translation initiation by displacing any inhibitory-bound complex (Parra-Palau et al., 2005; Ueda et al., 2004). Mnk activity is dependent on MAPK-mediated phosphorylation and eIF4G. To phosphorylate eIF4E and drive translation, MAPK-activated Mnk is required to first bind to eIF4G, which stabilizes Mnk and facilitates Mnk-phosphorylation of eIF4E S209 (Shveygert et al., 2010).

During inflammatory pain events, interleukin 6 (IL-6) and nerve growth factor (NGF) signaling activate nociceptor receptors and trigger intracellular signaling cascades to increase rapid protein synthesis through mTOR signaling (Melemedjian et al., 2010). At the heart of translational control in MAPK signaling, the Mnk-eIF4E interaction is a critical determinant for rapid protein synthesis involved in nociceptor sensitization (Moy et al., 2017). The post-mitotic nociceptors are susceptible to many different signaling pathways, but these studies suggest that the signaling pathways converging on eIF4E have a pivotal point in regulation of nociceptor sensitivity as shown in mammalian models.

Ion Channels Involved in Thermal and Mechanical Nociception

Conserved in sensory systems throughout metazoans, transient receptor potential (TRP) channels are involved in nearly all sensory modalities and contribute to many diseases when defective (Nilius and Owsianik, 2011). Of the TRP channels in *Drosophila*, the TRPA (TRP ankyrin) class is expressed in larval md-IV neurons and is involved in nociceptor function at basal and sensitized states (Boiko et al., 2017; Tracey et al., 2003; Zhong et al., 2012). The role of TRPA channels in nociception is strongly conserved as nociception and many pain conditions in mammals are regulated by TRPA channels (Brierley et al., 2011;

Kim et al., 2010; Wang et al., 2008). Specific to *Drosophila*, the TRPA channels *painless* and *trpa1* regulate thermal, mechanical, and chemical nociception; activation of TRPA channels in md-IV results in the larval NEL response (Tracey et al., 2003; Zhong et al., 2012).

Of the TRP channels, TRPA proteins are arguably the most diverse polymodal receptors as TRPA members are activated by warm temperatures (~32 °C), mechanical stimuli, and electrophilic reactive species such as allyl isothiocyanate (AITC) found in pungent compounds like wasabi, mustard oil, and horseradish (Bandell et al., 2004; Jordt et al., 2004; Zhong et al., 2012). Interestingly, chemical activation of TRPA1 by electrophilic reactive species in *Drosophila* and humans has been linked to ancient gustatory function for protection from toxic compounds (Kang et al., 2010).

Analysis of Painless isoforms revealed that the 8 ankyrin repeat domain of the protein are responsible for the thermal nociception, but not mechanical nociception, which suggests that noxious thermosensory and mechanosensory properties of Painless are independently coded for (Hwang et al., 2012). In support of the ankyrin-dependent thermosensation property in Painless, a separate study defined regions of TRPA1 isoforms encoded by 37 intracellular amino acids near the last ankyrin repeat and the N terminus are required for temperature activation (Zhong et al., 2012). Whereas calcium imaging in a heterologous cell system revealed alternatively spliced isoforms of *Drosophila* TRPA1 have various temperature thresholds (22-32 °C), expression of Painless in HEK293 cells revealed direct activation of Painless by noxious heat (Sokabe et al., 2008; Zhong et al., 2012).

Responsible for the generation and conduction of electrical signals in neurons, sodium voltage-gated ion channels (Na_v channels) and potassium voltage-gated ion channels

(K_v channels) are essential for neural signaling. When Na_v channels are pharmacologically blocked, all receptor and ion channel-induced currents are halted at the axon and further prevented from signaling to the secondary neuron (Binshtok et al., 2007). In *Drosophila* neurons, the sodium voltage-gated ion channel, *paralytic (para)*, is required for nociceptor activity- when *para* is depleted, nociceptors cannot fire action potentials and the NEL response to noxious stimuli is completely lost (Dyson and Bellemer, 2017; Lin et al., 2009).

Tight control of transcripts by degradation systems during plasticity is demonstrated by regulation of the voltage-gated sodium channel, Na_v 1.7, in human nociceptor sensitivity. The *SCN9A* gene encodes for Na_v 1.7 in humans and is targeted for degradation by micro-RNAs that cleave the transcript in the 3'-UTR (Shao et al., 2016). When micro-RNA expression was knocked-down under hypersensitive states, rat pain responses were exaggerated. (Shao et al., 2016). This demonstrates how disruptions in post-transcriptional regulation can lead to destabilized synapses and abnormal signaling transmission.

In *Drosophila*, post-transcriptional mechanisms apply to temporal regulation of *para* expression. The 3' UTR binding protein, Pumilio, binds to *para* mRNA with cofactors Nanos and Brain Tumor (Brat) in motor neurons to spatially repress and de-repress translation of *para* to maintain homeostatic regulation of neuron excitability (Muraro et al., 2008). Accordingly, when *pumilio* is knocked down in *Drosophila* nociceptors, larvae demonstrate a hypersensitive thermal nociception phenotype which suggests that Para expression is no longer repressed in a temporal manner to maintain homeostatic control (Dyson and Bellemer, 2017; Stewart and Bellemer, 2017).

Mechanisms of Drosophila Nociceptor Sensitization

The main questions that drive pain neurobiology ask, “which mechanisms control nociceptor sensitivity and how do those mechanisms change during injury-induced inflammatory states?” *Drosophila* has been established as a robust, genetically-tractable model to answer these questions through UV-induced epidermal tissue damage (Babcock et al., 2009). The model was built on the premise that human exposure to acute UV radiation results in epidermal damage (sunburn) and nociceptor sensitization. The model tested 2 types of sensitization phenotypes, allodynia and hyperalgesia, in thermal nociception assays at specific time intervals to determine the latency (measured in seconds) of the NEL response elicited at subthreshold (38 °C) and threshold (46 °C) was significantly different from the “mock” UV treated larvae (Babcock et al., 2009). This sensitization model is supported by mammalian research in that allodynia and hyperalgesia are induced after UV-injury in a time-dependent manner (Basbaum et al., 2009; Davies et al., 2005).

Similar to mammals, larval UV-induced sensitization is modulated by activity of cytokines released from apoptotic epidermal cells in damaged tissue (Babcock et al., 2009). Tumor Necrosis Factor α (TNF- α) cytokine signaling directly controlled thermal allodynia, but not thermal hyperalgesia, which suggests that thermal allodynia and hyperalgesia in *Drosophila* larvae are regulated by independent mechanisms (Babcock et al., 2009). A subsequent study in *Drosophila* larvae demonstrated that Hedgehog signaling not only works similarly to TNF- α signaling in thermal allodynia, but that Hedgehog is also required for thermal hyperalgesia (Babcock et al., 2011). Furthermore, Hedgehog signaling controls mammalian opioid receptor signaling which has significant implications for possible chronic pain therapies (Babcock et al., 2011).

Hedgehog signaling is classically known as an essential signaling pathway in temporal regulation of embryonic development (Hooper and Scott, 2005). Hedgehog signaling inhibits the Patched receptor by direct interaction, which relieves the inhibitory block from Smoothed to lead to intracellular signaling that culminates by the activation of canonical Hedgehog transcription factors, Cubitus interruptus and Engrailed (Babcock et al., 2011). Through nociceptor-specific knock-down of Hedgehog signaling components, larvae developed thermal allodynia and hyperalgesia after UV-induced injury and also through ectopic signaling (Babcock et al., 2011). Specifically, Hedgehog-induced nociceptor sensitization in *Drosophila* demonstrated that the Painless channel is involved thermal allodynia while the TRPA1 is involved with thermal hyperalgesia (Babcock et al., 2011). This suggests that Painless and TRPA1 function as downstream modulators of the Hedgehog signaling pathway to bidirectionally control nociceptor sensitivity at basal and sensitized states, independently. However, development of both Painless-dependent allodynia and TRPA1-dependent hyperalgesia required Hedgehog-stimulation transcription via Cubitus interruptus and Engrailed, which suggests that transcriptional regulation of Hedgehog signaling is critical during *Drosophila* nociceptor sensitization.

Characterization of RNA-Binding Proteins in Regulation of Nociceptor Sensitivity

Based on a genetic screen conducted by my predecessor, Amber Dyson, this project focused on characterizing genes that were determined to have a role in *Drosophila* nociception (Dyson and Bellemer, 2017). Through nociceptor-specific gene knock-down experiments in *Drosophila*, the thermal and mechanical nociception assays were utilized to characterize the role of RNA-binding proteins in nociceptor regulation. We hypothesized that

thermal and mechanical nociception analyses would show that RNA-binding proteins are required for nociceptor regulation at baseline and sensitized states.

An external genetic screen determined that RNA-binding proteins have extensive roles in shaping dendrite morphology of *Drosophila* md-IV neurons (Olesnick et al., 2014). However, the role of dendrite morphology and nociceptor function in *Drosophila* has not been directly linked (Babcock et al., 2011; Barbee et al., 2006). The effects of gene knock-down on nociceptor morphology were quantified to ensure that the nociception phenotypes were not just a side effect of maladaptive development. We hypothesized that dendritic arborization complexity would not be significantly impacted due to the weak link between defects in morphology and nociceptor function.

Though cytokine signaling and Hedgehog signaling, among a few other pathways, have been documented in regulation of nociceptor sensitivity, the RNA processing mechanisms that control nociceptor sensitivity at baseline states has yet to be elucidated. How is ion channel expression regulated to maintain baseline sensitivity, especially during ion channel endocytosis and degradation? Are there mRNPs that target ion channel transcripts for subcellular localization, thus local translation? Do the eIF4E isoforms differentially initiate translation in nociceptors to regulate sensitivity to noxious stimuli? If eIF4E isoforms do differentially initiate translation in nociceptors to regulate sensitivity, how might this be accomplished? Is it possible that mRNA transcripts contain *cis*-regulatory sequences in the 5' UTR or 3' UTR, similar to *cis*-regulatory sequences that control rapid dendritic localization of immediate early genes (IEGs) in hippocampal neurons (Mayford et al., 1996; Wu et al., 1998)?

Furthermore, there are gaps in the current understanding of the *Drosophila* nociceptor sensitization pathways. What genes are upregulated during Hedgehog signaling and TNF- α signaling, which genes are repressed, if any? Why is TNF- α signaling specific to the development of thermal allodynia, but not thermal hyperalgesia? Lastly, which nuclear RNA processing mechanism controls Hedgehog signaling? RNA processing mechanisms, including post-transcriptional regulation, controlled by RNA-binding proteins likely control *Drosophila* nociceptor sensitivity by maintaining regulation of temporal and spatial gene expression.

To investigate the mechanisms by which RNA-binding proteins function in thermal and mechanical nociception, four specific aims were designed. This thesis project focused on addressing each specific aim to better characterize nociceptor sensitivity regulation in *Drosophila*.

Specific Aims:

- I. Define baseline thermal and mechanical larval sensitivity when target genes are knocked down
- II. Quantify the effects of gene knockdown on nociceptor morphology
- III. Design sensitization assays and characterize the effect of gene knockdown on sensitization
- IV. Construct transgenic *Drosophila* that express Venus-fusion proteins for overexpression experiments and observation of subcellular localization

METHODS

Drosophila genetics

To test the roles of specific RNA-binding proteins in nociceptor function, cell-specific RNA interference (RNAi) was used to knock down transcript levels in the nociceptors. This was accomplished by utilizing the binary GAL4/upstream activation sequence (UAS) system is a genetic tool used to control cell-specific gene expression levels in *Drosophila* (Duffy, 2002). The *Drosophila pickpocket* (*ppk*) gene, which encodes for a Degenerin/Epithelial Sodium Channel (DEG/ENaC), demonstrated limited expression to the md-IV neurons thus was used to create a nociceptor-specific GAL4 driver (*ppk1.9-GAL4*) (Ainsley et al., 2003). The theorized advantage of cell-specific gene knock-down is that the cell is not deprived completely of the targeted protein, which likely allows for minimal functionality and increased chance of cell survival. However, the efficiency of cell-specific gene knockdown was not confirmed in this project, so the possibility exists that false-positive or negative phenotypes were observed due to incomplete knockdown or off-target effects. To decrease the possibility of these confounding factors, this project used *ppk-GAL4*; *UAS-dicer-2* combined with *UAS-RNAi* to increase gene knockdown efficiency (Dietzl et al., 2007).

To increase gene expression, *ppk1.9-GAL4* flies were crossed to flies with a UAS element upstream of the gene of interest, which resulted in increased transcription of the gene in nociceptors (Duffy, 2002). To decrease gene expression in nociceptors, *ppk1.9-GAL4*; *UAS-dicer-2* flies were crossed to *UAS-RNAi* lines for each gene of interest. RNAi disrupts mRNA transcripts to result in reduced levels of the target protein and the presence of *dicer-2*

increases transcript RNAi activity (Dietzl et al., 2007; Duffy, 2002). Fly stocks were obtained from Bloomington *Drosophila* Stock Center (Table 1).

For each gene knock-down experiment, 6 virgin females of the *w; ppk1.9-GAL4; UAS-dicer2* driver line were crossed with 3 males from the following RNAi stocks: *UAS-eIF4E-1 RNAi* (#34096), *UAS-eIF4E-3 RNAi* (#42804), *UAS-eIF4AIII RNAi* (#32444), *UAS-eIF4AIII RNAi* (#32907), *UAS-Barentsz RNAi* (#61165), *UAS-Barentsz RNAi* (#30482). To determine the effect of gene knock-down on dendritic morphology, md-IV neurons were visualized by crossing 6 virgin females of the *UAS-mCD8::GFP; UAS-dicer2* driver line with 3 males of the following RNAi lines: *UAS-eIF4AIII RNAi* (#32444), *UAS-Barentsz RNAi* (#61165), *UAS-Barentsz RNAi* (#30482).

Table 1. Fly genotypes used to investigate thermal and mechanical nociception.

Gene	Celera Gene (CG) #	RNAi Line
<i>eIF4E-1</i>	CG4035	BDSC 34096
<i>eIF4E-3</i>	CG8023	BDSC 42804
<i>eIF4AIII</i>	CG7483	BDSC 32444
		BDSC 32907
<i>Barentsz</i>	CG12878	BDSC 30482
		BDSC 61165

BDSC: Bloomington *Drosophila* Stock Center.

To account for each of the elements of the GAL4/UAS system, 2 negative controls were used. The *ppk-GAL4* (GAL4-only) negative control groups in all RNAi knockdown experiments were obtained by 6 virgin females of the *w; ppk1.9-GAL4; UAS-dicer2* driver line crossed to 3 males of the *y¹ v¹; Py[+t7.7]=CaryP}attP2* (BDSC# 36303). The *UAS* only negative control groups in all RNAi knockdown experiments were obtained by 6 *w¹¹¹⁸* virgin females crossed to 3 males of the previously listed RNAi lines. For over-expression of *eIF4AIII* in md-IV neurons, 6 virgin females from the *ppk1.9-GAL4* driver line were crossed to 3 males of the constructed *UAS-eIF4AIII::Venus* line (described in *Molecular Cloning and Transgenic Fly Construction* of Methods section). All flies were mated in vials containing cornmeal molasses food and dry yeast in a 25 °C incubator on a 12-hour light/dark cycle. Wandering 3rd instar F1 larvae were harvested for nociception assays.

General Behavioral Assay Preparation

The larval nervous system is fully developed at the 3rd instar stage (Zwart et al., 2013). Wandering 3rd instar larvae were collected from cross vials and transferred to a glass petri dish slightly lubricated with yeast water (a pinch dry Baker's yeast dissolved in ~ 2 mL dH₂O). Larvae were allowed 3-5 minutes to assimilate to the testing environment before all nociception assays began. Only mobile 3rd instar larvae were tested to avoid confounding variables in the data such as motor dysfunction or incorrect stage of neural development. Each larva was tested once to avoid habituated responses in thermal assays. To validate preliminary data, experiments were later replicated with both the samples and data analyses blinded to the researcher to avoid testing bias.

Thermal Assay

A soldering iron probe with a flat-edged tip was used to deliver the noxious heat stimulus. A Variac Variable Transformer controlled the temperature of soldering iron probe. A thermocouple apparatus (IT-23 thermistor and BAT-12 digital thermometer) connected to the tip of the iron probe monitored the temperature at all times. The chiseled tip (~6 mm) of the heated probe was applied longitudinal axis of the larval body wall for at least 15 seconds per trial. At least 50 animals were tested per genotype. Baseline thermal nociception assays were conducted at 46 ± 0.5 °C, a suprathreshold noxious temperature for *Drosophila* larvae (Tracey et al., 2003). The temperature was lowered to 42 ± 0.5 °C, a temperature by which wild-type larvae show slightly delayed nociception, to identify hypersensitive phenotypes in baseline thermal nociception assays (Tracey et al., 2003). All injury-induced sensitization experiments tested larval thermal nociception at 42 ± 0.5 °C. All thermal assays were recorded at 30 frames per second using a digital camera that is connected to a dissecting microscope. The thermal nociception responses produced were analyzed using Adobe Premiere Pro. The nocifensive escape locomotion (NEL) was quantified by the time (s) it took the larva to complete a full 360° rotation from the time the heated probe initially contacted the organism. The non-parametric Wilcoxon Rank-Sum Test was used to determine statistical significance in NEL responses between control groups and experimental groups. Bonferroni's correction was applied to correct the α value in experiments that compared multiple groups.

Mechanical Assay

A Von-Frey Filament (10 mm) calibrated to deliver 50 mN of mechanical force was used to deliver the noxious mechanical stimulus. The initial larval response to the mechanical stimulus was manually recorded in binary format (NEL=1, no NEL= 0). At least 98 samples were recorded per genotype. Statistical significance between control and experimental groups was determined using the Chi-Square test. Bonferroni's correction was applied to correct the α value in experiments that compared multiple groups.

UV Exposure Assay

Late 2nd instar larvae were selectively transferred from cross vials onto petri dishes lubricated with yeast water and were transferred to a dry petri dish using a fine-tip paint brush. The larvae were immediately moved to a UV Crosslinker for UV exposure of 18 mJ/cm² at 254 nm. Each step was replicated for the mock control, but the mock larvae were not exposed to UV light. After the experimental conditions were applied, the sample larvae were transferred to recovery medium, apple cider agar plates lightly coated with yeast paste (0.7mL dI H₂O and 0.5mL dry baker's yeast), in 60 mm disposable petri dishes. Nociceptor sensitivity of 3rd instar larvae was tested 8 hours post-UV induced injury in thermal nociception assays. The NEL latency (s) quantified as previously described. The NEL latencies were then sorted into categorical groups (fast= NEL < 7 s; slow= 7-14.9 s; no NEL/delayed NEL \geq 15s). The model was designed because our classically used model (non-parametric report of NEL latency < 11 s) did not reflect the trends of nociceptor sensitivity demonstrated post-UV injury. The Chi-Square test was used to determine statistical differences between UV and mock-UV groups of the same genotype.

Live-cell Imaging

For live-cell imaging of md-IV neurons, 3rd instar larvae were immobilized via ligation of the ventral nerve cord near the segment A3 using a hair. Ligation of the ventral nerve cord severed the connectivity of the motor neurons that branch off the ventral nerve cord which resulted in paralysis. Each larval sample was mounted in 100% glycerol between two coverslips and tiled z-stacks were obtained with a Zeiss LSM 880 confocal microscope. Nociceptors were manually isolated from neighboring neurons in Photoshop. The ImageJ Sholl Analysis application was used to determine dendritic complexity quantified through the number of interactions between the dendritic field and concentric shells that radiated at specified intervals (10 pixels) from the manually designated soma (Ferreira et al., 2014). Students t-test was used to determine statistical significance of number of dendritic interactions and mean dendritic interactions between genotypes.

Molecular Cloning and Transgenic Fly Construction

RNA isolated from *w¹¹¹⁸* flies per (Bogart and Andrews, 2006) served as the template for first strand synthesis of cDNA via reverse transcriptase reaction (SuperScript III First-Strand Synthesis SuperMix, ThermoFisher). Primers were designed to amplify full-length open reading frames (ORFs) of *eIF4A* and *eIF4AIII*. Forward and reverse primer design, *eIF4A*: 5'-ATGGATGACCGAAATGAGATACC-3'; 5'-TTAAATCAAATCGGCAATATTAGC-3'. Forward and reverse primer design, *eIF4AIII*: 5'-ATGGCGCGCAAGAATGCC-3'; 5'-TTAGATCAAGTCAGCCACGTTC-3'. The DH5 α competent *E. coli* cells used in transformation experiments were manually harvested by the researcher per New England Biolabs Molecular Cloning manual. Amplified full-length ORFs of *eIF4A* and *eIF4AIII* were cloned into the pENTR/d-TOPO vector using the pENTRTM /D-

TOPO® Cloning Kit and later recombined using the Gateway Cloning LR Clonase reaction kit into the pTVW vector (The *Drosophila* Gateway Collection). The final constructs contained a UAS element, the gene of interest (*eIF4A* or *eIF4AIII*), and a Venus epitope tag (similar to GFP tag). Complete sequencing and correct insert orientation was confirmed through Sanger Sequencing by Eton Bioscience. Sequenced constructs were injected into *Drosophila* embryos by BestGene Incorporated to create transgenic fly lines that expressed a copy of the protein-Venus fusion in addition to the endogenous gene product through p-element mediated transformation. These transgenic flies were created for overexpression experiments and future determination of subcellular protein localization patterns in nociceptors.

RESULTS

To better understand the role of post-transcriptional control by RNA-binding proteins in thermal and mechanical nociception, isoforms of *eIF4E* and EJC components *eIF4AIII* and *btz* were strategically knocked down in *Drosophila* nociceptors using the GAL4/UAS system and the cell specific driver, *ppk1.9*. In all experiments, 2 negative controls were used to identify the effect of gene knockdown on thermal or mechanical nociception. Each of the 2 negative controls tested 1 element of the GAL4/UAS system. The GAL4-only control, labeled *ppk/+*, expressed the *ppk1.9-GAL4; UAS-dicer-2* but no *UAS-RNAi*. The UAS-only control, labeled *UAS- "gene" RNAi/+*, expressed the *UAS-RNAi* for the gene of interest, but no *ppk1.9-GAL4; UAS-dicer-2*. For all thermal experiments, increased latency (s) indicated hyposensitivity whereas decreased latency (s) indicated hypersensitivity. For all mechanical experiments, increased response (% response) indicated hypersensitivity whereas decreased response (% response) indicated hyposensitivity.

EIF4E-1 is required for thermal nociception, but not mechanical nociception

To test the role of the *eIF4E-1* isoform in thermal nociception, *eIF4E-1* was specifically knocked down in larval nociceptors as previously described. Shown in Figure 3, *eIF4E-1* knockdown larvae were modestly hyposensitive to noxious heat (46 °C) with an average NEL latency of 3.28 (s) compared to the average latency of the GAL4-only negative control group, 2.35 (s), and compared to the UAS-only negative control group, 2.55 (s). The increased latency of *eIF4E-1* knockdown larval response to noxious thermal heat was significantly different from the GAL4-only control larval response and UAS-only control larval response (Wilcoxon Rank Sum Test and Bonferroni's Correction Post-Hoc Test, GAL4-only, p=0.002; UAS-only, p=0.009).

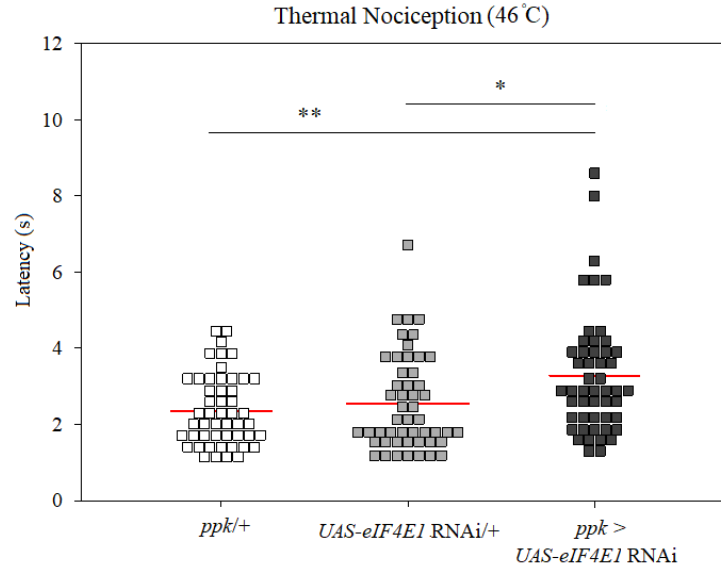


Figure 3. Nociceptor-specific knockdown of eIF4E-1 causes hyposensitive thermal nociception. Larvae with nociceptor-specific knockdown of eIF4E-1 showed a significantly longer latency to respond to a noxious thermal stimulus (46°C) than the GAL4-only and UAS-only controls. Response latencies of individual animals are plotted as points on the graph, while the mean for each genotype is indicated as a red horizontal bar. (n=50 for all groups; * $p \leq 0.025$ by Wilcoxon Rank-Sum Test and Bonferroni's Correction post-hoc test; ** $p \leq 0.005$ by Wilcoxon Rank-Sum Test and Bonferroni's Correction Post-Hoc Test; *** $p \leq 0.001$ by Wilcoxon Rank-Sum Test and Bonferroni's Correction post-hoc test).

To determine if eIF4E-1 has a role in regulation of nociceptor sensitivity to noxious mechanical stimuli, larvae with nociceptor-specific knockdown of *eIF4E-1* were subjected to 50 mN of force using a calibrated Von Frey filament. Mechanical nociception in *eIF4E-1* knockdown larvae was not affected by depleted levels of the cap-binding protein (Figure 4). The proportion of *eIF4E-1* knockdown larvae response to noxious mechanical stimuli was 42%, which was not statistically different from proportion of GAL4-only negative control larvae or UAS-only negative control larvae response to noxious mechanical stimuli, 51% and 51%, respectively (Chi Square Test, $p = 0.339$). Therefore, data suggests that eIF4E-1 has a role in larval nociceptor sensitivity to noxious thermal, but not noxious mechanical stimuli.

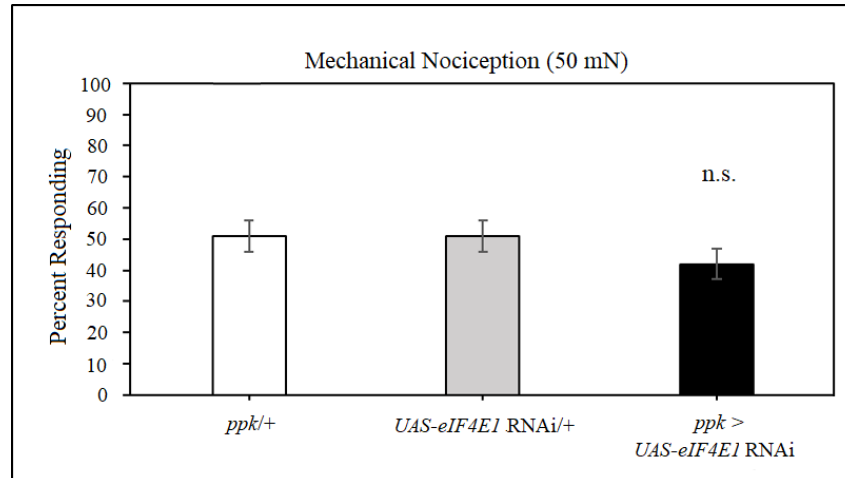


Figure 4. Knockdown of eIF4E-1 does not cause defects in mechanical nociception. A slightly smaller proportion of larvae with nociceptor-specific knockdown of eIF4E-1 responded to the mechanical stimuli (42%) than the GAL4-only control larvae (51%) and the UAS-only control larvae (51%). (n=100 for all groups; Chi-Square Test, n.s.= not significantly different). Bars indicate the proportion of animals from each genotype that responded to the first application of the mechanical stimulus. Error bars indicate the standard error of the proportion.

eIF4E-3 is not required for thermal or mechanical nociception

Protein alignment analysis revealed that eIF4E-3 does not contain a phosphorylation target residue at the classical *Drosophila* LK6 phosphorylation site (Figure 2). Due to the lack of this phosphorylation site, *Drosophila* eIF4E-3 may have a differential role in translation regulation in nociceptors. To determine if eIF4E-3 has a role in thermal nociception like eIF4E-1, nociceptor sensitivity of larvae with nociceptor-specific knockdown of *eIF4E-1* were subjected to noxious thermal heat (Figure 5). The thermal nociception assay revealed *eIF4E-3* RNAi larvae responded at similar rates as the GAL4-only negative control group and the UAS-only negative control group (average NEL listed respectively, 2.82 s, 2.62 s, 2.85 s). Thus, the effect of *eIF4E-3* RNAi on thermal nociception was not significantly different from the negative control groups (Wilcoxon Rank Sum Test and Bonferroni's Correction Post-Hoc Test, GAL4-only, p=0.659; UAS-only, p=0.939).

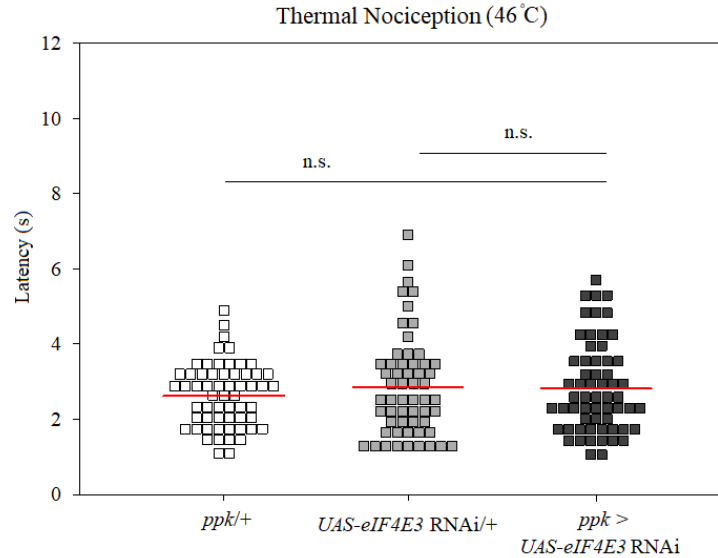


Figure 5. Nociceptor-specific knockdown of eIF4E-3 does not cause defects in thermal nociception. Larvae with nociceptor-specific knockdown of eIF4E-3 showed a significantly longer latency to respond to a noxious thermal stimulus (46°C) than the GAL4-only and UAS-only controls. Response latencies of individual animals are plotted as points on the graph, while the mean for each genotype is indicated as a red horizontal bar. (n=57 for all groups; n.s.= not significant by Wilcoxon Rank-Sum Test and Bonferroni's Correction post-hoc test).

To determine if eIF4E-3 has a role in regulation of nociceptor sensitivity to noxious mechanical stimuli, larvae with nociceptor-specific knockdown of *eIF4E-3* were subjected to 50 mN of force using a calibrated Von Frey filament. Mechanical nociception in *eIF4E-3* knockdown larvae was not affected by depleted levels of the cap-binding protein (Figure 6). The proportion of *eIF4E-3* knockdown larvae response to noxious mechanical stimuli was 59%, which was not statistically different from proportion of GAL4-only negative control larvae or UAS-only negative control larvae response to noxious mechanical stimuli, 51% and 60%, respectively (Chi Square Test, $p = 0.371$). Thus, data suggests that eIF4E-3 does not have a role in regulation of nociceptor sensitivity at baseline states.

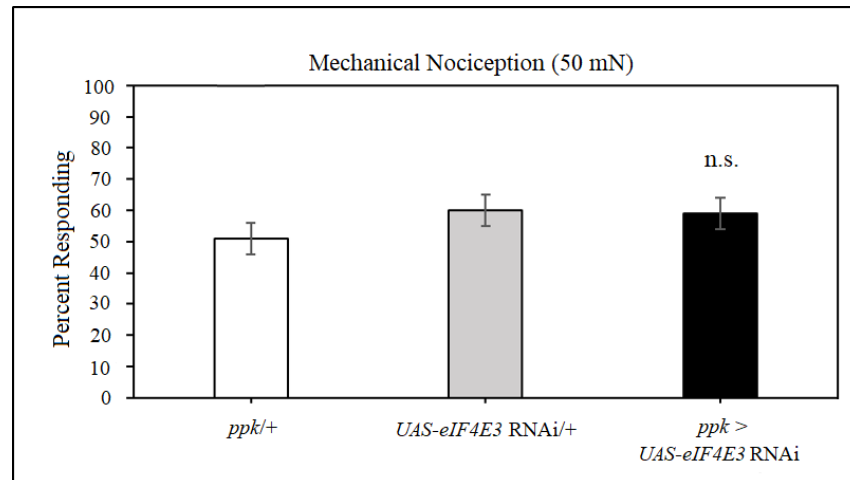


Figure 6. Nociceptor-specific knockdown of eIF4E-3 does not cause defects in mechanical nociception. A slightly smaller proportion of larvae with nociceptor-specific knockdown of eIF4E-3 responded to the mechanical stimuli (59%) than the GAL4-only control larvae (51%) and the UAS -only control larvae (60%). (n=100 for all groups; Chi-Square Test, n.s.= not significantly different). Bars indicate the proportion of animals from each genotype that responded to the first application of the mechanical stimulus. Error bars indicate the standard error of the proportion.

Knockdown of EJC core component produces severe hyposensitivity in thermal and mechanical nociception

As the RNA-binding protein of the EJC core, eIF4AIII coordinates splicing events for many transcripts, especially those with long introns (Roignant and Treisman, 2010). Notably, 2 separate *Drosophila* genetic screens have revealed that the nuclear EJC factors, eIF4AIII, Tsu, and Magoh have roles in splicing key transcripts that are involved in MAPK signaling and Hedgehog signaling (Ashton-Beaucage et al., 2014; Garcia-Garcia et al., 2017). Due to the important RNA-binding role of eIF4AIII in the EJC, transcripts required for thermal and mechanical nociception of *Drosophila* larvae may be impacted by depleted levels of eIF4AIII in nociceptors.

To validate and elaborate on Dyson's findings from the previously conducted genetic screen, the effect of nociceptor-specific knockdown of EJC core component, *eIF4AIII*, on thermal and mechanical nociception was determined (Dyson and Bellemer, 2017). Consistent

with Dyson's previous results, thermal nociception was severely impaired in *eIF4AIII* RNAi larvae. Both *eIF4AIII* RNAi lines of the attP2 background (#32907 and #32444) resulted in significant hyposensitive thermal and mechanical nociception compared to their respective negative controls.

As shown in Figure 7, nociceptor-specific knockdown of *eIF4AIII* (BDSC RNAi line #32444) significantly delayed the latency (s) by which larvae responded to noxious heat (average NEL= 6.18 s) compared to the negative control groups, GAL4-only (average NEL= 2.75 s), and UAS-only (average NEL= 2.95 s); Wilcoxon Rank Sum Test and Bonferroni's Correction Post-Hoc Test, GAL4-only, $p \leq 0.001$; UAS-only, $p \leq 0.001$ (Figure 7).

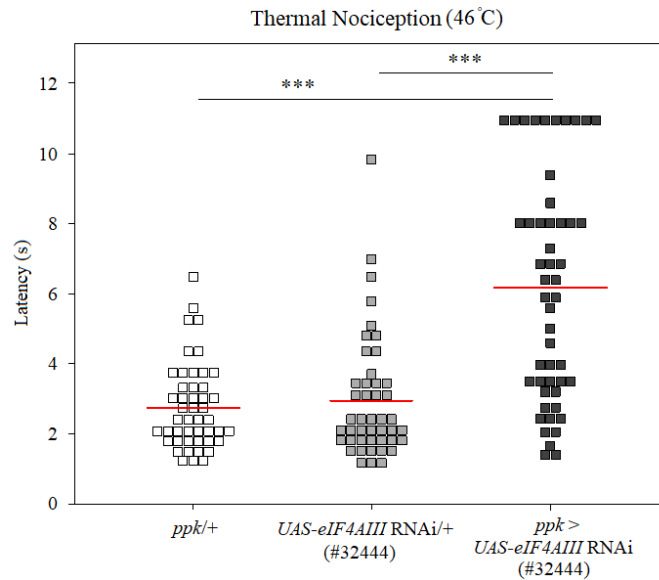


Figure 7. Nociceptor-specific *eIF4AIII* RNAi (#32444) causes severe hyposensitivity in thermal nociception. Larvae with nociceptor-specific knockdown of *eIF4AIII* (#32444) showed a significantly longer latency to respond to a noxious thermal stimulus (46°C) than the GAL4-only and UAS-only controls. Response latencies of individual animals are plotted as points on the graph, while the mean for each genotype is indicated as a red horizontal bar. (n=50 for all groups; *** $p \leq 0.001$ by Wilcoxon Rank-Sum Test and Bonferroni's Correction post-hoc test).

To test if eIF4AIII is also required for detection of noxious mechanical stimuli, eIF4AIII knockdown larvae were subjected to 50 mN of force using a calibrated Von Frey filament. Mechanical nociception assays revealed that knockdown of eIF4AIII in nociceptors causes significant defects in larval response to noxious mechanical stimuli. Furthermore, both eIF4AIII RNAi lines of the attP2 background (#32907 and #32444) resulted in significant hyposensitive mechanical nociception compared to their respective negative controls. Shown in Figure 8, the proportion of eIF4AIII knockdown larval response to noxious mechanical stimuli (15%) was significantly smaller than the proportion of GAL4-only negative control response (40%) and UAS-only negative control response (54%) (Chi Square Test, $p \leq 0.001$).

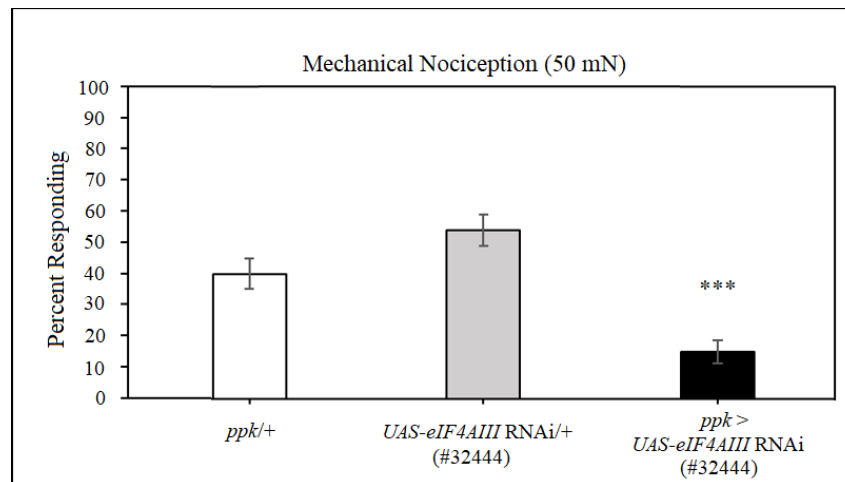


Figure 8. Nociceptor-specific eIF4AIII RNAi (#32444) causes severe hyposensitivity in mechanical nociception. A slightly smaller proportion of larvae with nociceptor-specific knockdown of eIF4AIII (#32444) responded to the mechanical stimuli (15%) than the GAL4-only control larvae (40%) and the UAS-only control larvae (54%). (n=100 for all groups; Chi-Square Test, *** $p \leq 0.001$). Bars indicate the proportion of animals from each genotype that responded to the first application of the mechanical stimulus. Error bars indicate the standard error of the proportion.

In support of the thermal hyposensitivity phenotype demonstrated by the eIF4AIII RNAi line #32444 (Figure 7), the eIF4AIII RNAi #32907 line significantly decreased larval sensitivity to noxious heat (Figure 9). eIF4AIII RNAi (#32907) larvae responded to noxious

thermal stimuli at a significantly delayed latency (average NEL= 5.67 s) compared to the negative control groups, GAL4-only (average NEL= 2.55 s), and UAS-only (average NEL= 2.56 s); Wilcoxon Rank Sum Test and Bonferroni's Correction Post-Hoc Test, GAL4-only, $p \leq 0.001$; UAS-only, $p \leq 0.001$ (Figure 9). This data suggests that the similar thermal hyposensitive phenotypes from both RNAi lines were not results of off-target effects of RNAi, rather that eIF4AIII participates in the regulation of thermal nociception.

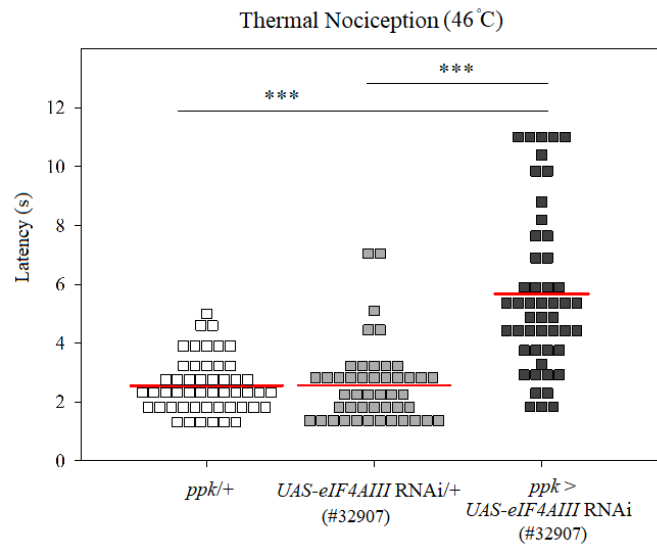


Figure 9. Nociceptor-specific eIF4AIII RNAi (#32907) causes severe hyposensitivity in thermal nociception. Larvae with nociceptor-specific knockdown of eIF4AIII (RNAi #32907) showed a significantly longer latency to respond to a noxious thermal stimulus (46°C) than the GAL4-only and UAS-only controls. Response latencies of individual animals are plotted as points on the graph, while the mean for each genotype is indicated as a red horizontal bar. (n=50 for all groups; *** $p \leq 0.001$ by Wilcoxon Rank-Sum Test and Bonferroni's Correction post-hoc test).

Similar to the hyposensitivity to noxious mechanical stimuli of *eIF4AIII* RNAi #32444 larvae, *eIF4AIII* RNAi #32907 larvae also demonstrated hyposensitivity to noxious mechanical stimuli. The proportion of *eIF4AIII* RNAi (#32907) larval response was significantly smaller (27%) than the proportion of GAL4-only negative control response

(44%) and UAS-only negative control response (57%) (Chi Square Test, $p \leq 0.001$) (Figure 10).

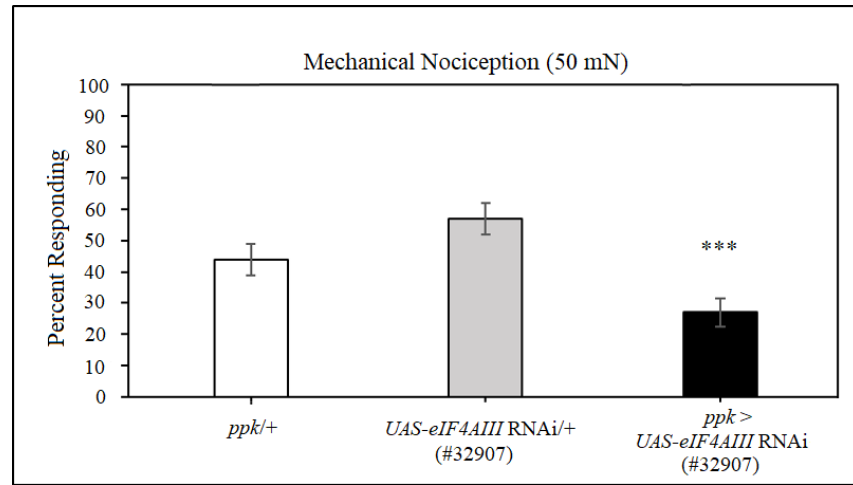


Figure 10. Nociceptor-specific eIF4AIII RNAi (#32907) causes severe hyposensitivity in mechanical nociception. A slightly smaller proportion of larvae with nociceptor-specific knockdown of eIF4AIII (#32907) responded to the mechanical stimuli (27%) than the GAL4-only control larvae (44%) and the UAS -only control larvae (57%). (n=100 for all groups; Chi-Square Test, *** $p \leq 0.001$). Bars indicate the proportion of animals from each genotype that responded to the first application of the mechanical stimulus. Error bars indicate the standard error of the proportion.

Due to the strong hyposensitivity phenotypes of *eIF4AIII* knockdown larvae to thermal and mechanical stimuli, it was hypothesized that overexpression of *eIF4AIII* would increase nociceptor sensitivity to noxious stimuli. This hypothesis was based on the idea that eIF4AIII may control EJC activity, thus nociceptor sensitivity, in a bidirectional fashion. To overexpression *eIF4AIII*, the constructed *UAS-eIF4AIII::Venus* transgenic fly line was used. Expression of *UAS-eIF4AIII::Venus* was driven in the nociceptors using the nociceptor-specific driver, *ppk1.9-GAL4*, which resulted in a copy of eIF4AIII::Venus additional to the endogenous copy of eIF4AIII.

Similar to the baseline thermal and mechanical nociception experiments, the nociception response of the experimental group was compared to the nociception response of a GAL4-only negative control and a UAS-only negative control. Unexpectedly,

overexpression of *eIF4AIII::Venus* resulted in hyposensitive thermal nociception (Figure 11), which was similar to the effect of *eIF4AIII* knockdown on thermal nociception (Figures 7, 9). The *eIF4AIII* overexpression larvae demonstrated a delayed response (average NEL latency 5.867 s) compared to the GAL4-only control (average NEL latency 3.25 s) and the UAS-only control (average latency 4.023 s). The delayed response of *eIF4AIII* overexpression larvae to noxious heat was significantly different from the 2 negative controls (Wilcoxon Rank Sum Test and Bonferroni's Correction Post-Hoc Test, GAL4-only, $p \leq 0.001$; UAS-only, $p \leq 0.001$). Thus, this data does not support the original hypothesis and suggests that eIF4AIII does not bidirectionally control nociceptor sensitivity.

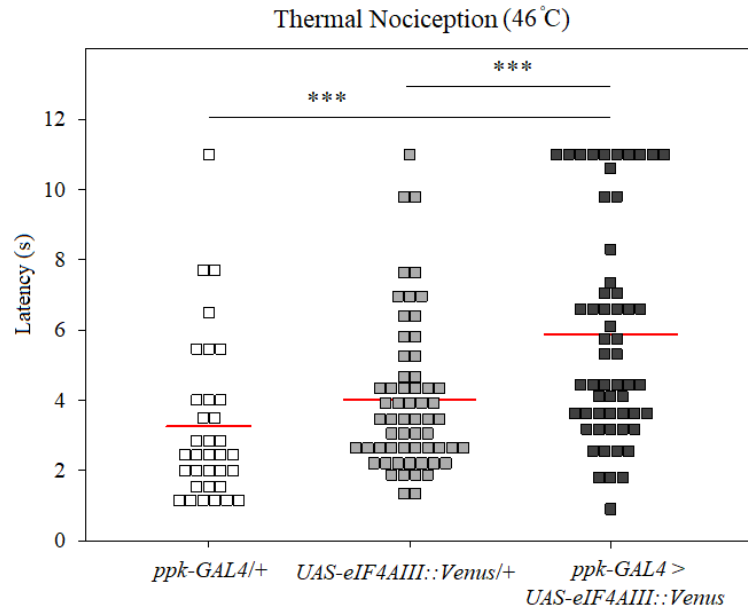


Figure 11. Nociceptor-specific overexpression of eIF4AIII causes severe hyposensitivity in thermal nociception. Larvae with nociceptor-specific overexpression of eIF4AIII showed a significantly longer latency to respond to a noxious thermal stimulus (46°C) than the GAL4-only and UAS-only controls. Response latencies of individual animals are plotted as points on the graph, while the mean for each genotype is indicated as a red horizontal bar (*ppk-GAL4* n= 34, *UAS-eIF4AIII::Venus* n=60, *ppk-GAL4 > UAS-eIF4AIII::Venus* n=57; *** $p \leq 0.001$ by Wilcoxon Rank-Sum Test and Bonferroni's Correction post-hoc test).

Knockdown of EJC core component, Btz, does not affect thermal nociception, but increases mechanical nociception

Due to the strong nociception phenotypes of *eIF4AIII* knockdown larvae, the EJC likely has a role in regulation of nociceptor sensitivity. But do individual EJC core components have specific roles in nociception? To investigate deeper into this question, the role of Btz in thermal and mechanical nociception was tested through nociceptor-specific knockdown of *btz*. The researcher was compelled to investigate the role of Btz in larval nociception due to the recently described interactions between Btz and eIF4AIII and stoichiometric relationship of the proteins in the cytoplasm (Chazal et al., 2013). Because Btz only joins the EJC before nuclear export, any effect of *btz* knockdown would likely result from affected post-transcriptional regulation of key thermal or mechanical transcripts.

To test if Btz has a role in the regulation of nociceptor thermal sensitivity, 2 RNAi lines from the same genetic background (attP2) were used. Knockdown of *btz* using the BDSC RNAi line #30482 did not affect nociceptor sensitivity to noxious heat (average NEL latency 2.89 s) compared to the negative control groups, GAL4-only (average NEL latency 2.69 s), and UAS-only (average NEL latency 2.45 s); Wilcoxon Rank Sum Test and Bonferroni's Correction Post-Hoc Test, GAL4-only, $p = 0.710$; UAS-only, $p = 0.477$ (Figure 12).

Similarly, knockdown of *btz* using the BDSC RNAi line #61165 did not affect nociceptor sensitivity to noxious heat (average NEL latency 3.54 s) compared to negative control groups, GAL4-only (average NEL latency 2.64 s) and UAS-only (average NEL latency 4.48 s); Wilcoxon Rank Sum Test and Bonferroni's Correction Post-Hoc Test, GAL4-only, $p = 0.026$; UAS-only, $p = 0.074$ (Figure 14).

Contrastingly, the proportion of *btz* RNAi larval response to noxious mechanical stimuli was higher than the proportional of GAL4-only and UAS-only larval response to noxious mechanical stimuli in *btz* RNAi #30482 larvae (73%, 57%, 52%, listed knockdown, GAL4-only, and UAS-only; Chi Square Test, $p = 0.021$; Figure 13). The same hypersensitivity phenotype to noxious mechanical stimuli was seen in *btz* RNAi #61165 larvae (61%, 45%, 46%, listed knockdown, GAL4-only, and UAS-only; Chi Square Test, $p = 0.040$) (Figure 15).

Overall, thermal and mechanical nociception analyses revealed that the role of Btz in nociception may be modality specific. Thermal nociception analyses demonstrated that *btz* is not required for regulation of thermal nociception at 46 °C (Figures 12, 14). However, when *btz* was knocked down specifically in the nociceptors, the larval response to noxious mechanical force (50 mN) was hypersensitive (Figures 13, 15). These nociception phenotypes were consistent in both *btz* RNAi lines tested (BDSC #61165 and BDSC #30482). Because the nociception phenotypes seen in both RNAi lines were similar, the effect of *btz* knockdown in those 2 RNAi lines are not likely due to off-target effects. Instead, the data suggests that knockdown of Btz caused defects in nociceptor sensitivity to noxious mechanical stimuli in *Drosophila* larvae.

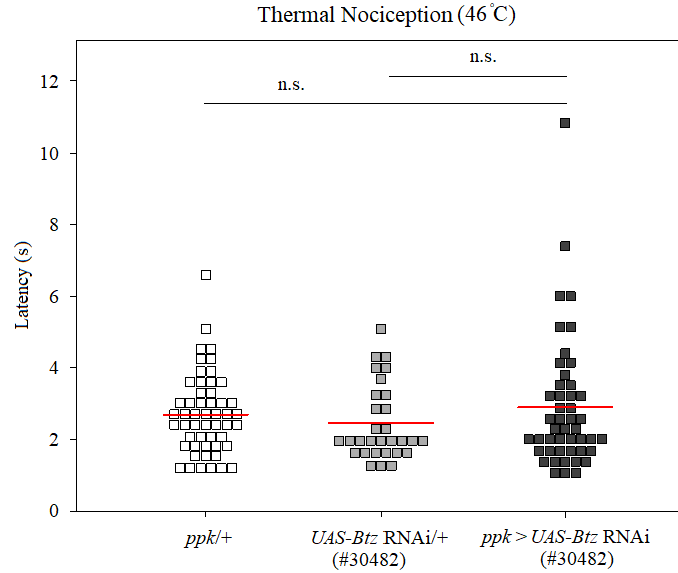


Figure 12. Nociceptor-specific Btz RNAi (#30482) does not cause defects in thermal nociception. Larvae with nociceptor-specific knockdown of Btz showed similar responses to noxious thermal stimulus (46°C) than the GAL4-only and UAS-only controls. Response latencies of individual animals are plotted as points on the graph, while the mean for each genotype is indicated as a red horizontal bar. (n=50 for all groups; n.s.= not significant by Wilcoxon Rank-Sum Test and Bonferroni's Correction post-hoc test).

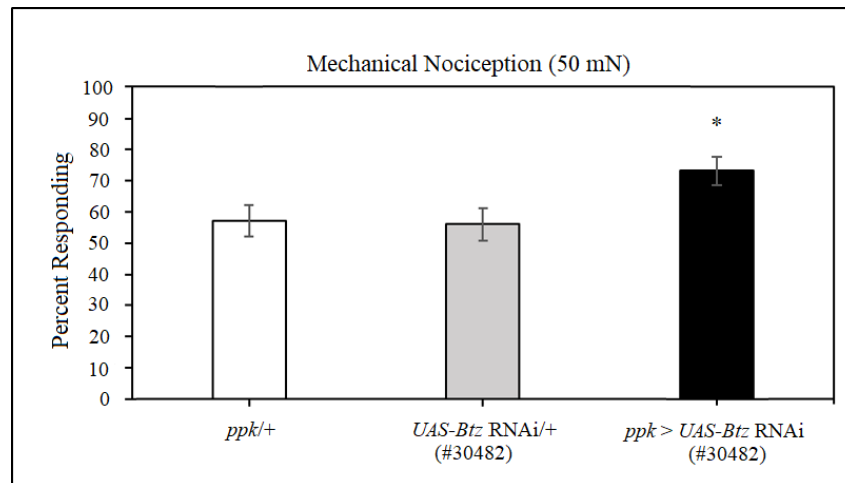


Figure 13. Nociceptor-specific Btz RNAi (#30482) causes hypersensitivity in mechanical nociception. A significantly larger proportion of larvae with nociceptor-specific knockdown of Btz responded to the mechanical stimuli (73%) than the GAL4-only control larvae (57%) and the UAS-only control larvae (52%). (n=100 for all groups; Chi-Square Test, * $p \leq 0.05$ by Chi Square Test). Bars indicate the proportion of animals from each genotype that responded to the first application of the mechanical stimulus. Error bars indicate the standard error of the proportion.

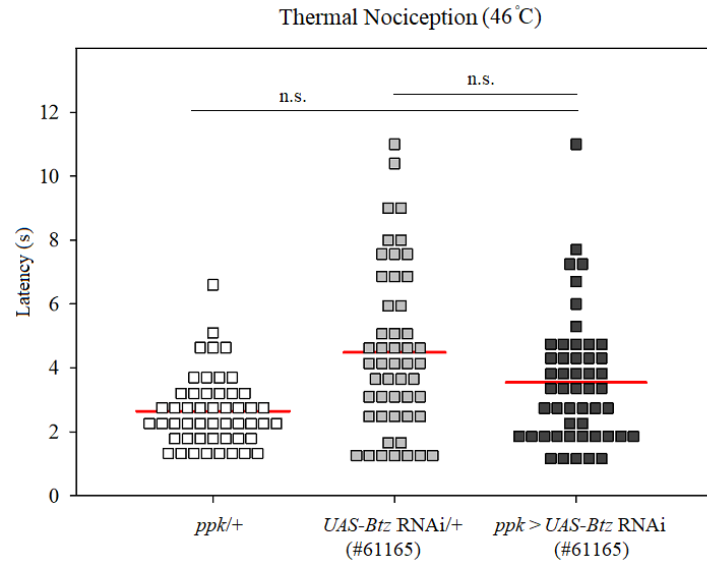


Figure 14. Nociceptor-specific Btz RNAi (#61165) does not cause defects in thermal nociception. Larvae with nociceptor-specific knockdown of Btz showed similar response latencies to noxious thermal stimulus (46°C) to the GAL4-only control and the UAS-only control. Response latencies of individual animals are plotted as points on the graph, while the mean for each genotype is indicated as a red horizontal bar. (n=50 for all groups; n.s.= not significant by Wilcoxon Rank-Sum Test and Bonferroni's Correction post-hoc test).

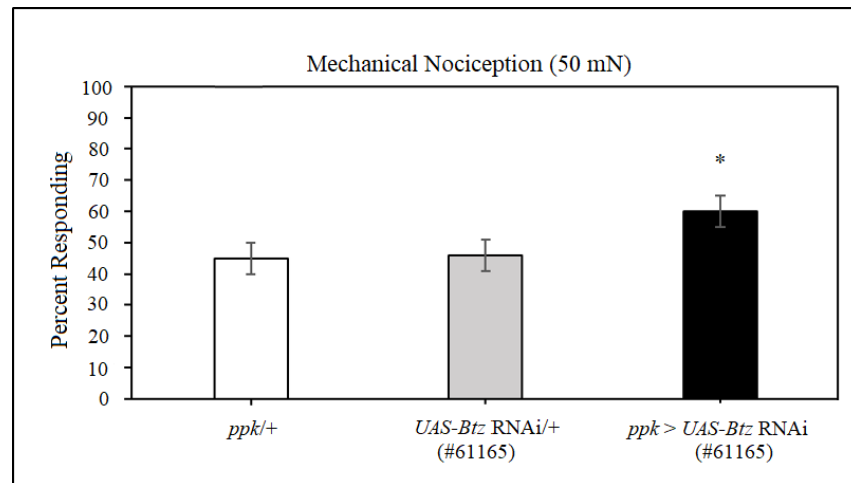


Figure 15. Nociceptor-specific Btz RNAi (#61165) causes hypersensitivity in mechanical nociception. A significantly larger proportion of larvae with nociceptor-specific knockdown of Btz responded to the mechanical stimuli (61%) than the GAL4-only control larvae (45%) and the UAS-only control larvae (46%). (n=100 for all groups; Chi-Square Test, * $p \leq 0.05$ by Chi Square Test). Bars indicate the proportion of animals from each genotype that responded to the first application of the mechanical stimulus. Error bars indicate the standard error of the proportion.

Dendritic Arborization Complexity is Not Significantly Impacted by Knockdown of EJC components

Previously, a genetic screen identified multiple RNA-binding proteins in control of md-IV dendrite morphogenesis (Olesnicky et al., 2014). Nociception defects may potentially arise from defects in md-IV morphogenesis. Therefore, it is important to measure md-IV morphology to determine whether major morphological defects are present. To determine if the deficiency in nociception of EJC core components stemmed from developmental defects, the expression of GFP and EJC knockdown in md-IV neurons was driven by *ppk1.9-GAL4>mCD8::GFP; UAS-dicer 2*. Live-cell imaging of *Drosophila* 3rd instar larvae revealed that *eIF4AIII* RNAi (n= 10) did not significantly affect the dendritic complexity of md-IV neurons in comparison to the dendritic complexity of wild-type md-IV neurons (n=10) (Figure 16). Dendritic complexity was quantified by the sum of dendritic interactions with the concentric shells of Sholl Analysis and by the average number of interactions. Though not statistically different from wild-type morphology (sum of interactions= 22561 interactions, average interactions= 23.04), *eIF4AIII* RNAi dendritic morphology was slightly reduced (sum of interactions= 17068 interactions, average interactions= 18.09); Sum of Dendritic interactions, p= 0.191 by Students t-test; Average Dendritic interactions, p= 0.174 (Figure 16).

The other focal EJC component, Btz, was investigated to determine the effects of *btz* RNAi on nociceptor morphology. Similar to *eIF4AIII* RNAi effects on dendritic morphology, *btz* RNAi did not cause significant defects in dendritic morphogenesis (n= 5). However, in contrast to the reduced dendritic complexity caused by *eIF4AIII* RNAi, *btz* RNAi slightly increased dendritic complexity (Figure 17). The sum of dendritic interactions of wild-type

neurons and *btz* RNAi neurons were calculated to have 26079 and 30353 interactions, respectively (Students t-test, $p = 0.113$). The average number of dendritic interactions of wild-type neurons and *btz* RNAi neurons were calculated to be 26.31 and 30.63 interactions, respectively (Students t-test, $p = 0.113$).

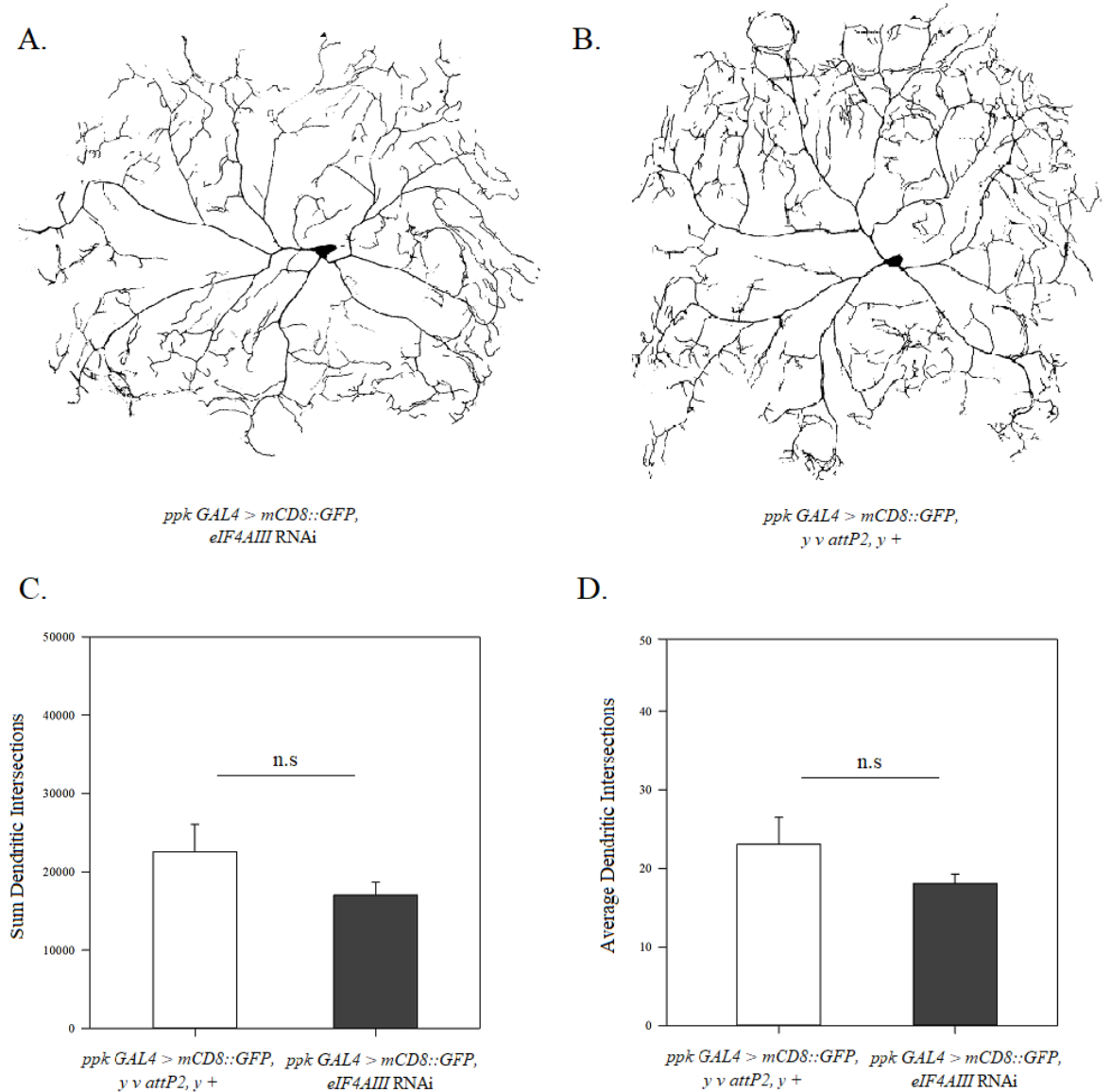


Figure 16. Depleted levels of eIF4AIII does not affect mdIV morphology. Analysis of live-cell imaging of md-IV neurons. A) Representative image of eIF4AIII RNAi md-IV morphology, B) Representative image of wild-type md-IV morphology. C) Sum of dendritic intersections determined by Sholl analysis. Sum of dendritic intersections of eIF4AIII RNAi md-IV neurons was not statistically different from the sum of dendritic intersections of wild-type md-IV neurons ($n = 10$; Students t-test, $p = 0.174$). D) Average dendritic intersections of eIF4AIII RNAi md-IV neurons was not statistically different from the average dendritic intersections of wild-type md-IV neurons ($n = 10$, Students t-test, $p = 0.191$).

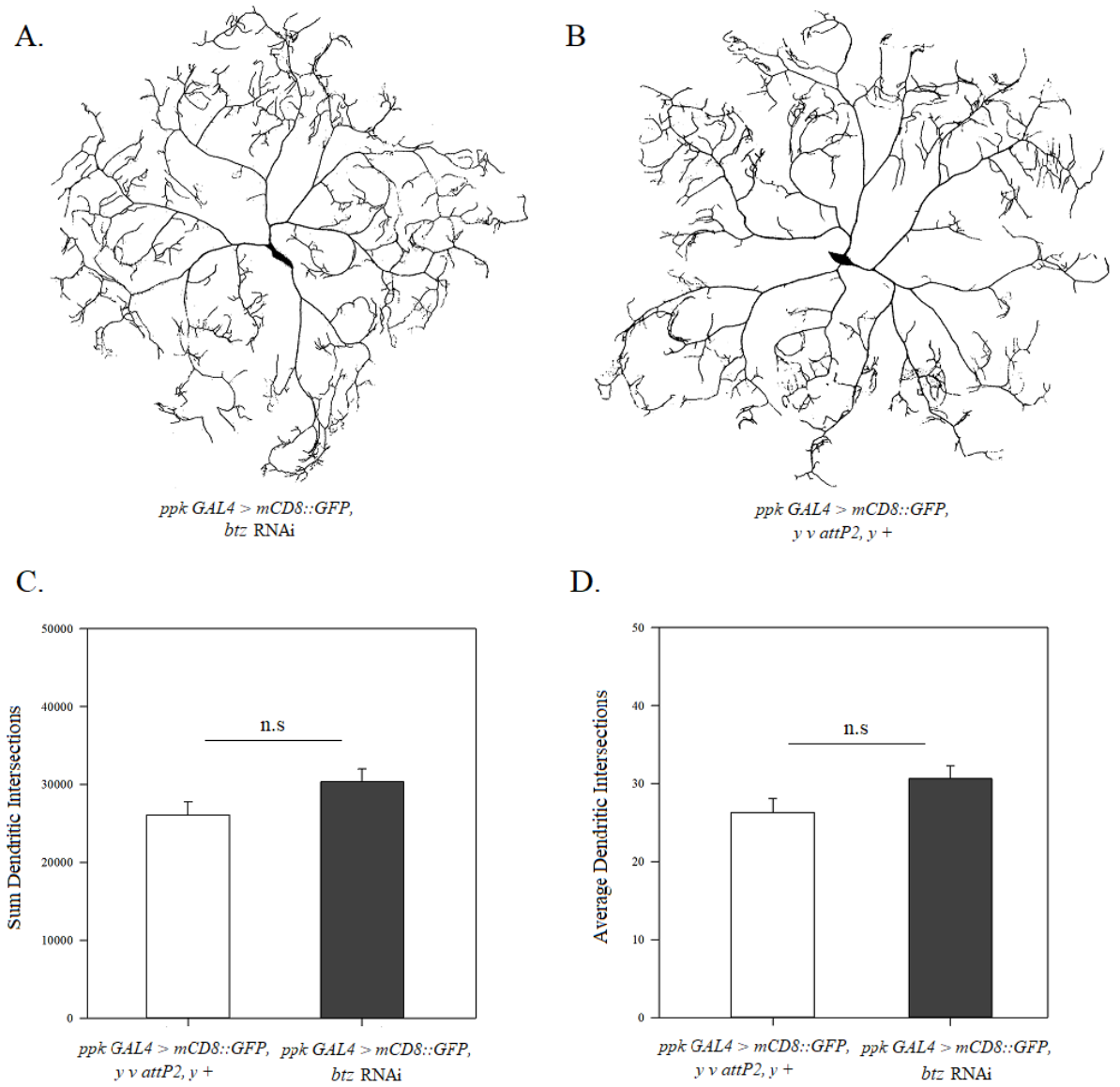


Figure 17. Depleted levels of Btz does not significantly affect mdIV morphology. Analysis of live-cell imaging of md-IV neurons A) Representative image of *btz* RNAi md-IV morphology, B) Representative image of wild-type md-IV morphology. C) Sum of dendritic intersections determined by Sholl analysis. Sum of dendritic intersections of *eIF4AIII* RNAi md-IV neurons was not statistically different from the sum of dendritic intersections of wild-type md-IV neurons (n= 5, Students t-test, p= 0.113). D) Average dendritic intersections of *eIF4AIII* RNAi md-IV neurons was not statistically different from the average dendritic intersections of wild-type md-IV neurons (n= 5, Students t-test, p= 0.113).

Nociceptor Sensitization via UV-induced Injury

To determine if eIF4AIII has a role in *Drosophila* sensitization, experiments based on the UV-injury induced model were conducted (Babcock et al., 2009). The effect of *eIF4AIII* knockdown and overexpression on nociceptor sensitization was tested. The data shown here was based on a preliminary model for nociceptor sensitization post-UV injury, as described in the methods. Nociceptor sensitivity of 3rd instar larvae was tested 8 hours post-UV induced injury in thermal nociception assays at 46 °C. The NEL latency (s) quantified as previously described. The NEL latencies were then sorted into categorical groups (fast= NEL < 7 s; slow= 7-14.9 s; no NEL/delayed NEL ≥ 15 s).

For each genotype, there was an experimental group and mock UV-exposed group. Like the previously designed experiments, the UV-induced nociceptor sensitization experiments used *ppk1.9-GAL4* only controls, *UAS* only controls, and *ppk-GAL4>UAS* experimental groups. Unlike previously designed experiments, only statistical comparisons of the same genotype were made, the proportion of larval response rate of negative control groups was not compared to the larval response rate of the experimental group. The development of hyperalgesia was determined through statistical analysis of the UV-exposed larvae (UV +) and mock-exposed larvae (UV -) from the same genotype.

To determine if larval nociceptors could still develop hyperalgesia after UV exposure if eIF4AIII was depleted, the UV-induced injury model was applied to *eIF4AIII* knockdown larvae (Figure 18). In the GAL4-only group, the UV-exposed larvae developed hyperalgesia to some extent as demonstrated by the fast responders group, but not the slow responders group (Chi-square test, fast p=0.022, slow p=0.099). Though the UV-exposed larvae responded at a higher rate than the mock-UV larvae of the UAS-only group, the UV-exposed

larvae did not respond at a significantly higher rate than the mock-UV larvae (Chi square test, Chi-square test, fast $p=0.176$, slow $p=0.100$, no response $p=0.219$). The *eIF4AIII* knockdown group developed hyperalgesia as demonstrated by the fast responders group and the non-responders group (Chi-square test, fast $p=0.022$, slow $p=0.099$, no response $p=0.003$). These data suggest that larval nociceptors are still capable of regulating sensitivity to noxious heat after injury when *eIF4AIII* is depleted. However, it is notable that *eIF4AIII* knockdown larvae respond to noxious heat at a markedly reduced rate than the negative control larval groups do.

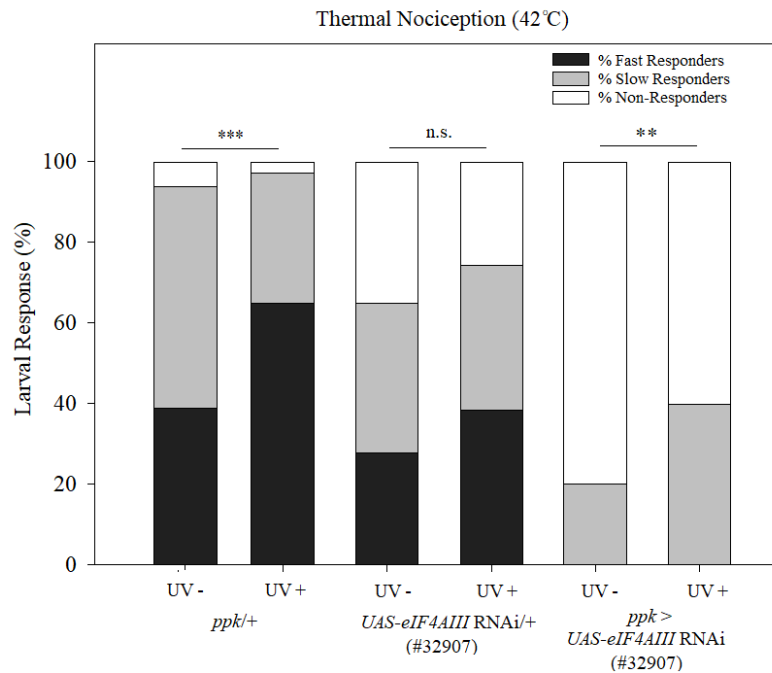


Figure 18. Nociceptor sensitization is decreased when *eIF4AIII* is knocked down, post-UV injury. Categorical NEL responses of *ppk*-GAL4 only (UV- $n=33$, UV+ $n=40$) were significantly different in 1 response group (Chi-square test, fast $p=0.022$, slow $p=0.099$). Categorical NEL responses of *UAS-eIF4AIII*:Venus only (UV- $n=43$, UV+ $n=39$) were not significantly different in any response groups (Chi-square test, fast $p=0.176$, slow $p=0.100$, no response $p=0.219$). Categorical NEL responses of *ppk > UAS-eIF4AIII*:Venus (UV- $n=20$, UV+ $n=20$) were significantly different in 1 response group (Chi-square test, fast $p=0.022$, slow $p=0.099$, no response $p=0.003$). Statistical significance indicated, n.s.= not significantly different by Wilcoxon Rank Sum Test; * $p \leq 0.05$ by Wilcoxon Rank-Sum Test; ** $p \leq 0.01$ by Wilcoxon Rank-Sum Test; *** $p \leq 0.001$ by Wilcoxon Rank-Sum Test.

To determine if overexpression of *eIF4AIII* has an effect on nociceptor regulation after an UV-induced injury, the UV-induced injury model was applied to *ppk1.9-GAL4 > UAS-eIF4AIII::Venus* larvae (Figure 19). In the GAL4-only group, the UV-exposed larvae developed hyperalgesia as demonstrated by the fast responders group and the non-responders group, but not the slow responders group (Chi-square test, fast $p=0.014$, slow $p=0.880$, no response $p=0.005$). The UV-exposed larvae of the UAS-only group responded at a significantly higher rate than mock-UV larvae as shown by all 3 categorical response groups (Chi-square test, fast $p=0.004$, slow $p=0.017$, no response $p<0.001$). Unlike the *eIF4AIII* knockdown larvae, the *eIF4AIII* overexpression larvae exposed to UV light did not develop hyperalgesia 8 hour after UV exposure (Chi-square test, fast $p=0.245$, slow $p=0.805$, no response $p=0.514$). These data suggest that nociceptors require balanced levels of eIF4AIII in order to regulate nociceptor sensitivity after UV injury.

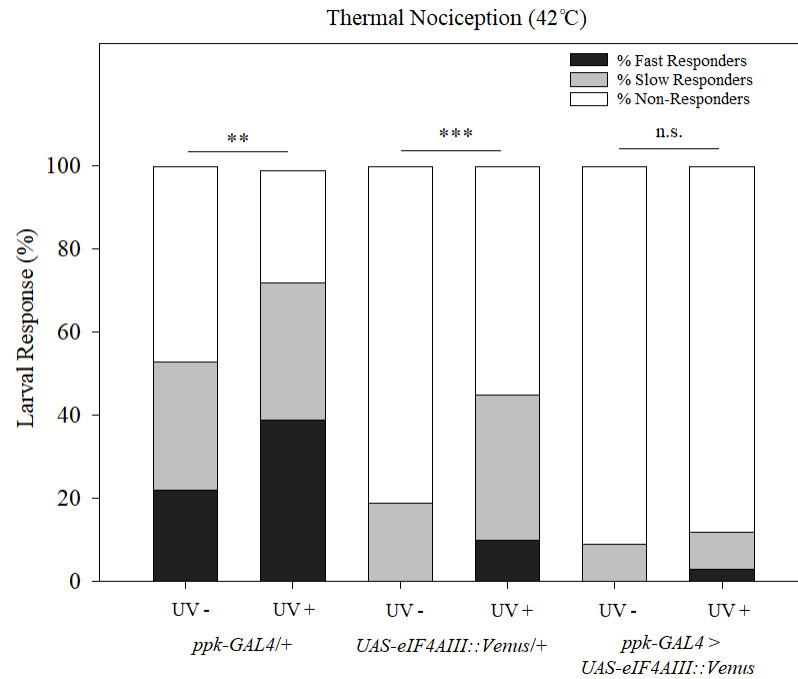


Figure 19. Nociceptor sensitization does not develop when eIF4AIII is overexpressed, post-UV injury. Categorical NEL responses of *ppk-GAL4* only (UV- n=32, UV+ n=33) were significantly different in 2 response groups (Chi-square test, fast p=0.014, slow p=0.880, no response p=0.005). Categorical NEL responses of *UAS-eIF4AIII::Venus* only (UV- n=16, UV+ n=20) were significantly different in all response groups (Chi-square test, fast p=0.004, slow p=0.017, no response p< 0.001). Categorical NEL responses of *ppk > UAS-eIF4AIII::Venus* (UV- n= 23, UV+ n=32) were not significantly different in all response groups: fast, slow, none (Chi-square test, fast p=0.245, slow p=0.805, no response p= 0.514). Statistical significance indicated, n.s.= not significantly different by Wilcoxon Rank Sum Test; *p≤0.05 by Wilcoxon Rank-Sum Test; **p≤0.01 by Wilcoxon Rank-Sum Test; ***p≤0.001 by Wilcoxon Rank-Sum Test.

DISCUSSION

Activity of RNA-binding proteins in RNA processing and translation controls nociceptor thermal and mechanical sensitivity at multiple points of gene expression. A summary of the observed nociception phenotypes is listed in Table 2. Larval hyposensitivity to noxious thermal heat was observed when *eIF4E-1* and *eIF4AIII* were knocked down. In no case were larval nociceptors shown to be hypersensitive to thermal heat. Larval sensitivity to noxious mechanical stimuli was not affected when *eIF4E* isoforms were knocked down. Though this result suggests that eIF4E isoforms do not participate in regulation of mechanical nociception, it does suggest that eIF4E isoforms may have specific roles in thermal nociception.

Larval hyposensitivity to noxious mechanical stimuli was observed when *eIF4AIII* was knocked down. In contrast, larval hypersensitivity to noxious mechanical stimuli was observed when *btz* was knocked down. In dendritic morphology analyses, neither knockdown of *eIF4AIII* nor *btz* resulted in significant morphology defects; however, trends of dendritic complexity were observed in the knockdown nociceptors. Parallel to the larval sensitivity levels to noxious mechanical stimuli, *btz* knockdown increased dendritic complexity whereas *eIF4AIII* knockdown decreased dendritic complexity. Thus, despite the lack of statistical significance, dendritic morphology may have an impact on nociceptor sensitivity. This observation is supported by a separate study which noted that branching patterns of md-IV neurons in *Drosophila* were associated with hyper- or hyposensitive thermal nociception phenotypes (Honjo et al., 2016). Because dendritic morphology is not always associated with defective nociception, a definitive conclusion on the effect of abnormal dendritic morphology and nociceptor sensitivity cannot be made. In order to definitively determine if the

nociception phenotypes produced by EJC knockdown experiments are due EJC regulatory mechanisms instead of defective morphogenesis, temporal control of nociceptor-specific gene knockdown would have to be conducted in the future (McGuire et al., 2003).

Table 2. Summary of the nociception phenotypes observed in this project. Thermal significance determined by Wilcoxon Rank-Sum Test and Bonferroni's Correction post-hoc test. Mechanical significance determined by Chi Square test.

Gene	RNAi Line	Thermal Phenotype	Thermal Significance	Mechanical Phenotype	Mechanical Significance
<i>eIF4E-1</i>	BDSC	Hyposensitive	p= 0.002 (GAL4-only)	None	p= 0.339
	34096		p= 0.009 (UAS-only)	distinguishable	
<i>eIF4E-3</i>	BDSC	None	p= 0.659 (GAL4-only)	None	p= 0.371
	42804	distinguishable	p= 0.939 (UAS-only)	distinguishable	
<i>eIF4AIII</i>	BDSC	Hyposensitive	p ≤0.001 (GAL4-only)	Hyposensitive	p ≤0.001
	32444		p ≤0.001 (UAS-only)		
<i>eIF4AIII</i>	BDSC	Hyposensitive	p ≤0.001 (GAL4-only)	Hyposensitive	p ≤0.001
	32907		p ≤0.001 (UAS-only)		
<i>eIF4AIII</i> Over-expression	---	Hyposensitive	p ≤0.001 (GAL4-only)	---	---
			p ≤0.001 (UAS-only)		
<i>Barentsz</i>	BDSC	None	p= 0.710 (GAL4-only)	Hypersensitive	p= 0.021
	30482	distinguishable	p= 0.477 (UAS-only)		
<i>Barentsz</i>	BDSC	None	p= 0.026 (GAL4-only)	Hypersensitive	p= 0.040
	61165	distinguishable	p= 0.074 (UAS-only)		

Differential Role of eIF4E isoforms in Nociception Regulation

The formation of condition-specific eIF4F complexes is considered to differentially control protein expression levels (Ho and Lee, 2016). However, data presented here does not provide evidence that thermal nociception in *Drosophila* is regulated by unique eIF4F complexes. Thermal nociception was hyposensitive when *eIF4E-1* was knocked down but thermal nociception was not affected by knockdown of *eIF4E-3* (Figures 3, 5). Data suggests that eIF4E-1 regulates of nociceptor function, likely due its role in translation initiation. But how does the knockdown of *eIF4E-1* exert the decreased thermal sensitivity effect? Is it possible that eIF4E-1 distinctively targets transcripts involved in thermal nociception?

The answer to the questions posed in the previous paragraph may lie in the mRNA code. Though eIF4E is primarily involved in translation initiation, human eIF4E has been demonstrated to increase export of nuclear mRNAs by interacting with a 50-nucleotide *cis*-regulatory element in the 3' UTR, but not through interaction with the 5' cap (Culjkovic et al., 2005, 2006). This 3' UTR *cis*-regulatory element is referred to as the 4E-sensitivity element and is present in transcripts that are specifically translated by eIF4E, like cyclin D1 (Culjkovic et al., 2005). Thus, eIF4E directly upregulates expression of its target mRNAs by increasing their nuclear export (Culjkovic et al., 2006). In a *Drosophila* genetic screen, Tracey and colleagues used laser capture microdissection and microarray analyses to identify nociceptor enriched genes involved in thermal nociception (Honjo et al., 2016). In the future, it would be extremely interesting to determine if any of the identified thermal nociception genes contain 4E-sensitivity elements, or *cis*-regulatory elements that function similarly in *Drosophila*, in their 3' UTR (Honjo et al., 2016).

Mechanical data suggest that transcripts encoded for noxious mechanical-transducing ion channels do not rely on eIF4E-1 for initiation of protein synthesis. It is possible that protein expression of transcripts encoded for mechanical nociception channels are regulated by other eIF4E isoforms, but knockdown of *eIF4E-3* did not cause a defect in mechanical nociception either. However, if mechanical nociception does not rely on cap-dependent translation, it is intriguing to consider the alternative possibilities that could explain this result. One possibility is that mechanical nociception is regulated through cap-independent translation. However, there is no evidence that cap-independent translation through IRES domains occurs in sensory neurons. Another existing possibility is that the synthesis of mechanical ion channels is dependent on the nuclear cap-binding proteins. Nuclear cap-binding proteins coordinate pre-mRNA splicing, nuclear export, and the pioneer round of translation either immediately after nuclear export, or after mRNP localization (Maquat et al., 2010). Further investigation into the role eIF4E isoforms and other cap-binding proteins in noxious mechanosensory regulation is required.

Protein sequence alignments of eIF4E isoforms revealed that *Drosophila* eIF4E isoforms have varying phosphorylation sites (Figure 2) and binding motifs (Zuberek et al., 2016). This pattern suggests that *Drosophila* eIF4E isoforms are differentially regulated by signaling pathways, such as MAPK and mTOR. At basal cell states, varying phosphorylation sites of eIF4Es may not play a significant role in protein synthesis. However, these varying phosphorylation sites of *Drosophila* eIF4E isoforms may play a significant role in nociceptor sensitization and chronic nociceptor plasticity given that phosphorylation of this site on eIF4E has been demonstrated to be critical in the development of acute and persisting hypersensitivity in mice (Moy et al., 2017; Moy et al., 2018).

Dysregulated synaptic plasticity during nociceptor sensitization is a widely accepted theory for the development of chronic pain. Mnk-driven translation has been demonstrated to be negatively regulated by human eIF4E-3, which does not contain a Mnk-phosphorylation site (Landon et al., 2014). In *Drosophila*, eIF4E-3 does not have a LK6-phosphorylation site which suggests that during nociceptor sensitization, eIF4E-3 could function as a negative plasticity regulator since eIF4E-3 has stronger binding affinity for the 5' cap and can also compete with eIF4E-1 for 5' cap binding (Zuberek et al., 2016). Though it has been postulated by numerous studies, phosphorylation of eIF4E-1 does not increase 5' cap binding affinity (Landon et al., 2014). Future experiments investigating isoform-specific eIF4F complexes in *Drosophila* nociceptors during basal and sensitized cell states are required to validate this hypothesis.

Nociceptor Function and Sensitivity is Controlled by eIF4AIII

eIF4AIII is a core component of the EJC which operates in splicing events and post-transcriptional regulation. Loss-of-function data presented here suggest that eIF4AIII critically regulates nociceptor sensitivity to noxious thermal and mechanical stimuli. Without the function of eIF4AIII in nociceptors, *Drosophila* larvae cannot produce the NEL response in the same time wild-type larvae can, which could be attributed to dysregulated RNA splicing or RNA processing.

Furthermore, nociceptor function is sensitive to the level of eIF4AIII expression. Based on the hyposensitive nociception phenotype of *eIF4AIII* RNAi larvae, it was hypothesized that overexpression of *eIF4AIII* would not result in a hyposensitive phenotype, rather it would result in a hypersensitive nociception phenotype. Thermal analysis of nociceptor-specific *eIF4AIII* overexpression did not support the hypothesis, as *eIF4AIII*

overexpression resulted in larval hyposensitivity to noxious thermal stimuli. The hyposensitive phenotype from the overexpression data suggests that nociceptors require a precise level of eIF4AIII expression to maintain homeostasis. Interestingly, researchers focused on the role of the EJC in neurodevelopmental disorders have proposed that the “proper dosage” of EJC components is necessary for neural processes, which is in agreement with the *eIF4AIII* knockdown and overexpression data reported here (McMahon et al., 2016).

In the preliminary UV-injury induced sensitization experiments, both knockdown and overexpression of *eIF4AIII* resulted in large drops in the proportion of larval response to noxious thermal heat. The *eIF4AIII* knockdown larvae demonstrated nociceptor sensitization post-UV injury, which indicates that nociceptors are still able to sensitize in presence of residual eIF4AIII levels. Interestingly, nociceptor sensitization was lost in larvae that overexpressed eIF4AIII through the eIF4AIII::Venus copy. There are a few possibilities for this: 1) the cell does not have precise levels of eIF4AIII which leads to cellular dysfunction, 2) the eIF4AIII-Venus fusion inadvertently exerts a dominant negative effect in that the protein is too large to modulate mRNA nuclear export; therefore, the nucleus is overcrowded with mRNA transcripts and subcellular gene expression is disrupted, or 3) the energetic demands of increased nuclear export exhausts the cellular machinery which leads to overcrowding of mRNAs in the nucleus, cellular dysfunction, and disrupted subcellular gene expression. Though it is very plausible that the second suggestion is the cause of this result, preliminary imaging of live cells demonstrated that though eIF4AIII-Venus fusion is localized in the nucleus, the fusion protein was also localized to dendritic and axonal sites (data not shown). Therefore, either the first or third possibilities, perhaps even a combination of both, are likely the cause of the nociception phenotype. Overall, the sensitization data

should be considered lightly given that the data collected was based on a preliminary model that was designed post-analysis. At the time of the experiment, the protocol had just been successfully established in the lab and has since been optimized.

The EJC is Significant for Regulating Nociceptor Sensitivity

Depleted levels of EJC core proteins have opposing effects on nociceptor sensitivity when knocked down independently — depleted eIF4AIII resulted in thermal and mechanical hyposensitivity and depleted Btz resulted in mechanical hypersensitivity. This result was intriguing because it emphasized the different roles of the EJC binding partners. Literature supports the critical role of eIF4AIII in co-transcriptional and post-transcriptional RNA regulation whereas cytoplasmic Btz functions in mRNA localization and modulation of translation efficacy (Boehm and Gehring, 2016). Notably, Btz binds to mRNA transcripts to form translational-repressor mRNPs to localize mRNA and preferentially targets EJC-spliced targets for regulation (Fritzsche et al., 2013; Hauer et al., 2016). To unpack the large implications of the role of Btz in post-transcriptional regulation of neuronal gene expression, the EJC-regulated transcript, Arc (Activity regulated cytoskeletal associated protein), is used as an example.

Arc is a critical protein in synaptic plasticity and is highly regulated by multiple *cis*-regulatory elements in the 5' and 3' UTRs, which direct *Arc* synaptic localization immediately after synaptic plasticity events (Huang et al., 2003; Kang and Schuman, 1996). Because the exerted homeostatic effects of Arc activity must be tightly regulated, the *Arc* transcript harbors 2 introns in the 3' UTR which enables the EJC to trigger NMD after the pioneer round of translation (Giorgi et al., 2007). Thus, translation-dependent NMD of *Arc* occurs local to the activated synapse and is modulated by EJCs bound to the *Arc* 3' UTR

(Giorgi et al., 2007). To put the post-transcriptional roles of Btz into context, biochemical analyses determined that *Arc* transcripts always co-purify with Btz in dendritic granules, which suggest that *Arc* localization and translation is influenced by Btz activity (Fritzsche et al., 2013). Furthermore, a study that initially set out to investigate the effect of splicing on *Arc* dendritic expression observed that translation efficacy of the luciferase reporter in the *Arc* 3' UTR was significantly increased, which was an effect that was dependent on splicing of 3' UTR introns (Paolantoni et al., 2018). The mechanisms by which *Arc* is spliced, regulated by NMD, and efficiently translated due to splicing, are all consistent with the roles of the EJC in regulation of *Arc* and also support the role of Btz in preferentially upregulating translation efficacy of spliced-transcripts (Chazal et al., 2013; Giorgi et al., 2007).

It is plausible that the hypersensitive nociception phenotype of *btz* knockdown larvae demonstrated here is caused by unregulated translation of transcripts encoded for noxious mechanosensory ion channels. This postulation implies that noxious mechanosensory ion channels, like TRPA1 or Painless, may be regulated by the nuclear and cytoplasmic EJC components since Btz preferentially regulates spliced transcripts bound by the EJC. This situation would also be supported by the hyposensitive thermal and mechanical nociception phenotypes of *eIF4AIII* knockdown larvae. **If** the thermal and mechanical sensing TRPA channel, Painless, is regulated by the EJC, then depleted levels *eIF4AIII* would result in decreased functional transcripts and detection of noxious stimuli would be defective. The mode by which *eIF4AIII* would act as a determinant for functional transcript expression, thus overall protein abundance, would not be a novel mechanism as depletion of *eIF4AIII* has already been demonstrated to eradicate *mapk* functional transcripts and MAPK protein

abundance in *Drosophila* (Ashton-Beaucage et al., 2014; Ashton-Beaucage et al., 2010; Roignant and Treisman, 2010).

Proposed Models for EJC Regulation of Nociceptor Sensitivity

The data presented in this project suggest that the EJC controls nociceptor sensitivity likely through regulation of of key transcripts either during co-transcriptional RNA processing, post-transcriptional regulation, and/or translation. Given the robust role of the EJC in regulation nociceptor sensitivity, it is highly likely that the EJC plays a role in canonical nociceptor signaling pathways. Here, I suggest two new hypotheses for basal and sensitized nociceptor signaling, summarized in Figure 20. First, I propose that the EJC functions upstream of Ras/MAPK signaling by modulation of splicing of *mapk* and temporally controlled local translation. Secondly, I propose that the EJC serves as an unidentified modulator of Hedgehog signaling by controlling expression of the canonical Hedgehog signaling transcriptional factor, Cubitus interruptus, to control *Drosophila* nociceptor sensitization (Garcia-Garcia et al., 2017).

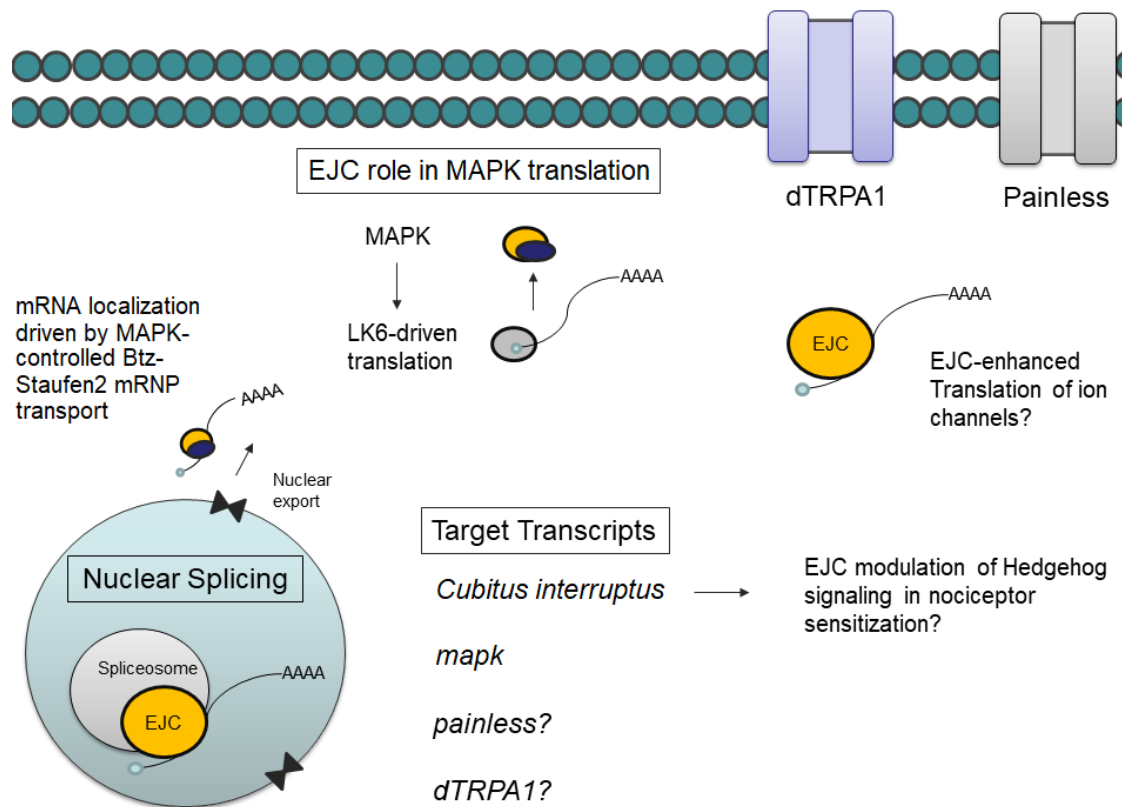


Figure 20. Graphical representation of the proposed mechanism by which the EJC may regulate nociception. The EJC likely controls gene expression in nociceptors at multiple RNA-processing levels. At the co-transcriptional level, the EJC modulates splicing events for a key transcript in Hedgehog signaling, *Cubitus interruptus*, which likely modulates nociceptor sensitization. The EJC may also modulate the Hedgehog pathway of nociceptor sensitization if the EJC regulates splicing events of *painless* and *dTRPA1*. Cytoplasmic EJC may also temporally and spatially control ion channel translation, which would control nociceptor sensitivity. Separate from the Hedgehog pathway, Btz-Staufen2 mRNP particles likely depend on MAPK modulation of motor proteins for mRNA localization. The Btz-Staufen2 mRNPs may also control translation in a spatial and temporal manner that is dependent on MAPK-LK6 signaling.

Under normal circumstances, eIF4AIII orchestrates *mapk* splicing and functional expression (Ashton-Beaucage et al., 2014; Ashton-Beaucage et al., 2010; Roignant and Treisman, 2010). The MAPK pathway modulates the motor proteins that fuel Staufen2-mRNP transport of mRNAs to dendritic sites (Jeong et al., 2007). Functional MAPK enables mRNA localization to distal dendritic sites through direct modulation of Staufen2 mRNPs via the MAPK docking site on Staufen2 (Nam et al., 2008). In the EJC-MAPK hypothesis, I propose that dendritically localized mRNA transcripts are held in translational-inhibitory

Btz-Staufen2 mRNPs until MAPK signaling activates LK6-phosphorylation of eIF4E to displace the inhibitory complex and activate dendritic translation (Bramham et al., 2016; Fritzsche et al., 2013). In summary, MAPK protein expression, controlled by eIF4AIII-modulated splicing, positively regulates mRNA localization and translation via modulation of Btz-Staufen2 mRNP activity. If this mechanism were in fact at play, it would put the EJC in a pivotal position for regulation of dendritic translation.

A *Drosophila* loss-of-function genetic screen uncovered that depleted levels of the nuclear EJC core components, eIF4AIII, Tsu, and Mago, all reduced Hedgehog signaling, but depleted Btz did not affect Hedgehog signaling (Garcia-Garcia et al., 2017). Nuclear EJC components were determined to regulate *Cubitus interruptus* protein abundance through modulation of *Cubitus interruptus* splicing (Garcia-Garcia et al., 2017). Unexpectedly, these data may provide the missing puzzle piece for a proposed model of Hedgehog signaling in *Drosophila* nociceptor sensitization (Babcock et al., 2011). *Cubitus interruptus* was required for the development of both thermal allodynia and hyperalgesia modulated by Painless and TRPA1 activity (Babcock et al., 2011). Because the EJC is now understood to regulate Hedgehog signaling via control of *Cubitus interruptus* expression levels, the EJC may regulate nociceptor sensitivity by indirectly regulating expression levels of Painless and TRPA1. However, if the EJC has a role in modulation of Painless and dTRPA1 splicing as previously discussed, Btz could function to enhance translational efficacy of the nociception ion channels during Hedgehog signaling (Figure 20).

Nociceptor Sensitization and Plasticity- How this project impacts future research

Homeostatic balance of protein synthesis is a critical process in post-mitotic cells. To delicately control gene expression of subcellular compartments in neurons in a spatial and

temporal manner, local translational machinery is required to rapidly respond to activity-dependent signaling. The data reported here poses new avenues to explore to better understand nociceptor regulation. The differential role of the eIF4Es in regulation of nociceptor function and the presence isoform-specific eIF4F complexes in nociception have yet to be understood. Importantly, it is unclear why mechanical nociception does not depend on regulation by eIF4E-1 or eIF4E-3. Further investigation is required to determine if other *Drosophila* eIF4E isoforms control mechanical nociception and how the proposed isoform selectivity would be conserved in mammalian mechanical nociception. It would also be interesting to determine if there is a difference in regulation of nociceptor sensitivity by eIF4E-1 and eIF4E-3 during post-injury sensitization events since eIF4E-3 cannot be regulated by LK6.

The EJC has a powerful influence on nociceptor function. Given the opposite nociception phenotypes of the EJC binding partners eIF4AIII and Btz, the EJC bidirectionally regulates nociceptor sensitivity, likely due to control of key nociception transcripts. Future experiments should characterize the role of the other two EJC components, Tsu and Mago. Because these proteins function as a heterodimer it would be interesting to determine the individual effect of depleted Tsu or Mago levels on nociception and also the effect of dual Tsu-Mago knockdown on nociception. Because the EJC serves a large array of responsibilities in development, dual Tsu-Mago knockdown in nociceptors should be constructed in the temporal restricting gene knockdown system. An excellent method to do this would be through the use of *ppk1.9 GAL4-GAL80; UAS-dicer-2*. When flies that express both GAL4 and GAL80 are grown at restrictive temperatures, GAL80 represses GAL4 activity, thus represses increased or decreased gene expression. However,

when the GAL4-GAL80 flies are moved to the permissive temperature for GAL4 activity, GAL80 is inactive, and GAL4-driven gene expression proceeds (McGuire et al., 2003). Therefore, by temporally controlling gene knockdown, neural development in *Drosophila* larvae is not influenced by depleted protein levels.

MAPK signaling has significant control of synaptic plasticity in learning and in chronic pain syndromes. Therefore, the EJC could possibly be in a position that controls synaptic plasticity and chronic pain syndromes by indirect control of MAPK signaling. The EJC does not regulate synaptic plasticity in manner simply defined as positive or negative regulation; rather, the EJC regulates key plasticity transcripts that have numerous effects on synaptic plasticity. Notably, the EJC preferentially regulates translation of transcripts that were spliced but does not regulate transcripts that encode ribosomal subunits, as shown by individual nucleotide resolution and immunoprecipitation (iCLIP) analyses of the mammalian transcriptome (Hauer et al., 2016).

To determine if EJC-control of *mapk* expression acts as an upstream regulator of translational expression rates, future experiments could investigate if translation is decreased when *eIF4AIII* is knocked using cell-specific translating ribosome affinity purification (TRAP) techniques. TRAP is a strong tool for RNA sequencing (RNAseq) determination of cell-specific translomes (Thomas et al., 2012). To analyze the transcripts that are translated in specific cell types, expression of a ribosomal protein tagged with a fluorescent protein is driven by cell-specific GAL4 drivers (like *ppk1.9-GAL4*) (Thomas et al., 2012). Introduction of the translational inhibitor, cycloheximide, stalls active ribosomes so that polysome immunoprecipitation followed by RNAseq can determine the cell-specific translome (Thomas et al., 2012). Therefore, this technique could be used to determine if translation of

certain transcripts, or even global translation, are affected by knockdown of core EJC components.

Future experiments focused on defining how Hedgehog signaling is regulated at the transcriptional level will be necessary to determine if the EJC modulates Hedgehog signaling to control nociceptor sensitization in *Drosophila* larvae. By uncovering the signaling events that modulate Hedgehog signaling, a whole new class of pharmacological targets will be available for testing (Babcock et al., 2011). In light of the current healthcare climate in the United States, defining the mechanisms that control opioid dependence is of the utmost importance for the nation's health and well-being.

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Appendix A

To construct a transgenic fly line that could spatially and temporally control gene expression, the *ppk1.9 GAL4-GAL80; UAS-dicer-2* fly line was constructed. Both GAL4 and GAL80 are located on the second chromosome of the fly genome. First, female *w; ppk1.9-GAL4* and male *w; tb-GAL80* were mated, then F1 virgin females were collected. The F1 virgin females were crossed to a balancer line, (*BoB*), and progeny were positively selected for eye color, which was indicative of the presence of GAL4 and GAL80 on the same allele (*ppk-GAL4/tb-GAL80*). Confirmation that progeny had both GAL4 and GAL80 was achieved through PCR confirmation of extracted DNA using primers that amplified sections of GAL4 and GAL80. A stable stock of the *ppk1.9 GAL4-GAL80* was constructed through subsequent generations using balancer lines.

Construction of this fly line was significant because GAL80 can temporally control GAL4 activity in a temperature-dependent manner. When flies that express both GAL4 and GAL80 are grown at restrictive temperatures, GAL80 represses GAL4 activity, thus represses increased or decreased gene expression. However, when the GAL4-GAL80 flies are moved to the permissive temperature for GAL4 activity, GAL80 is inactive, and GAL4-driven gene expression proceeds (McGuire et al., 2003). Therefore, by temporally controlling gene knockdown, neural development in *Drosophila* larvae is not influenced by depleted protein levels. This fly line can be used in future experiments focused on determining whether certain nociception phenotypes are due to defective nociceptor morphogenesis or defective cellular mechanisms when genes are knocked down.

Vita

Katherine Hoffman was born in 1993 in Raleigh, North Carolina to Elizabeth Beaney Liner and Gary Hoffman. She graduated from Leesville Road High School in Raleigh in June 2012. The following fall, she attended Appalachian State University and received a Bachelor of Science degree with a concentration in Cell and Molecular Biology and a minor in Chemistry in 2016. The following January, she continued her studies and conducted neurobiology research as a master's student at Appalachian State University. In December 2018, she was awarded with a Master of Science degree and was inducted into the Cratis D. Williams Society of Outstanding Graduate Students.

Katherine Hoffman has pursued further education and training as a biomedical researcher at a location that has yet to be determined. She works in Raleigh at the University of North Carolina as a neurobiology research assistant. She currently lives with her dog in Raleigh, sometimes Winston-Salem, and looks forward to living in the mountains again.